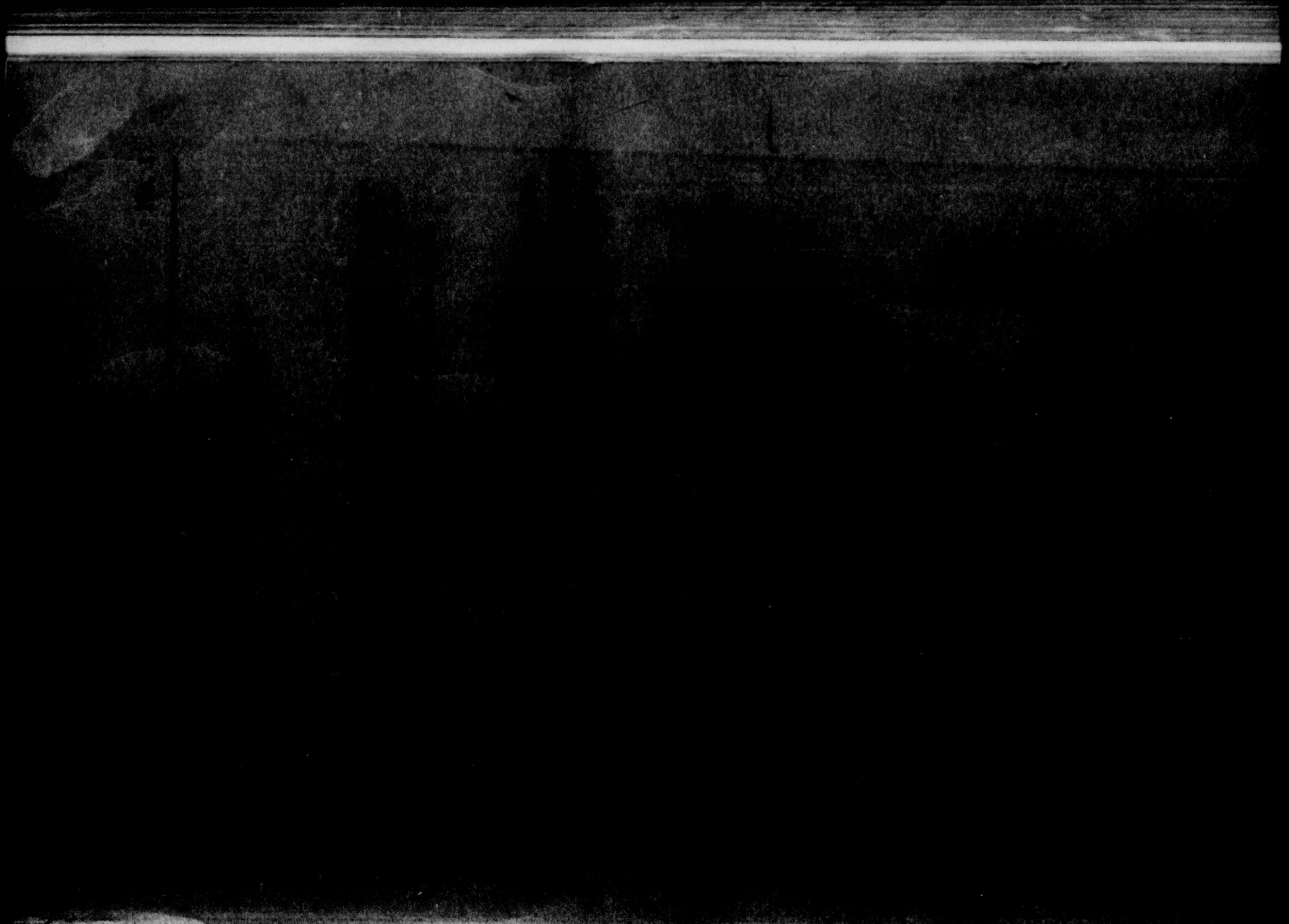

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Collaborator on the Journal of Biological Chemistry

Born	January 25, 1864
Died	September 23, 1916

THE STUDY OF CERTAIN DIETARY CONDITIONS BEARING ON THE PROBLEM OF GROWTH IN RATS.

By CASIMIR FUNK.

In Cooperation with Joseph Poklop.

(From the Huntington Fund, Memorial Hospital and Loomis Laboratory, and the Harriman Research Laboratory, Roosevelt Hospital, New York.)

(Received for publication, August 12, 1916.

During the progress of a series of investigations carried on by the writer in collaboration with Dr. Macallum,[1] it was deemed advisable to note in a series of control experiments the influence exerted by the addition of various specific substances, which might, not only from the standpoint of nutrition, but also from the standpoint of correcting deficiencies in diet, bring about a better condition of the experimental animals. The results obtained, covering a period of nearly 2 years and representing selected data noted by observation of several hundred rats experimented upon, are herewith presented.

As a matter of experience it might be stated at this time that the animals furnished to us by dealers seemed to lack that power of resistance which would make them suitable for our feeding experiments. This was manifested by a loss of hair on being placed in the metabolism cages, and early death before our experiments were well under way. This difficulty was promptly eliminated when rats bred in our laboratory under proper dietary conditions were substituted. The animals which were used for breeding purposes thrive best when kept on a diet of oats, bread, condensed milk, and yeast. The success of this diet was evidenced by the production of better animals from the physical, as well as from the experimental standpoint, than are produced by any other ways yet brought to our notice. Details of this dietary are shown elsewhere in the present paper.

[1] Funk, C., and Macallum, A. B., *J. Biol. Chem.*, 1916, xxvii, 51, 63.

In sequence to our earlier studies as to the value of oats as an exclusive diet for guinea pigs and rabbits,[2] our later experiments tend to show that oats, whether in seed or germinated, in conjunction with sodium bicarbonate or alone, were also an insufficient or unsuitable diet for young rats.

Our experiments show that the quantity of vitamines necessary for stimulating growth in rats is by no means small. If yeast is added to the diet to the extent of 1 per cent the rats grow for a short time, after which they begin to decline. Experiments which are not recorded in this paper have shown that at least 3 per cent of yeast is necessary to insure a satisfactory growth in rats. Still further experiments have shown that yeast has more effect in promoting growth than an addition of a few cc. of milk, as used by Hopkins.[3] Yeast can be regarded as a complete food by itself. It was therefore necessary to ascertain whether the good results obtained with this addition are not merely due to a correction of the nutritive value of the protein used (in this case, casein) or to the presence in it of nucleic acid. Consequently a diet was prepared in which the total casein nitrogen was substituted by yeast nitrogen. The results obtained were not as satisfactory as when yeast was used in smaller amounts for its vitamine content only, and not for nutritive value.

As orange juice has shown its value as a preventative of scurvy in rats, its value in promoting growth had to be ascertained. A diet which was made with orange juice instead of yeast was found to be without value for growing rats.

Finally an inquiry into the value of Lloyd's reagent as a precipitant for the growth-promoting substance in yeast was attempted. This reagent has been introduced by Seidell[4] for a quantitative separation of beri-beri vitamine from autolyzed yeast. He claimed that an addition of 50 gm. of the reagent to a liter of autolyzed yeast is sufficient for this purpose. Our results seem to show that a complete separation of the growth substance could not be effected, since both the precipitate and the filtrate were active, although to a lesser degree than the original solution, the activity of the precipitate being far more

[2] Funk, *J. Biol. Chem.*, 1916, xxv, 409.
[3] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.
[4] Seidell, A., *U. S. Public Health Report*, No. 325, 1916.

marked than that of the filtrate. We are not prepared to state at present whether our findings were due to the use of an insufficient amount of the reagent, or whether the separation of the growth-promoting factor into two fractions would be an explanation. We may be able to make a more definite statement in the near future. That the value of autolyzed yeast for growth experiments, as well as its antiscorbutic properties, for rats, is diminished by the above process, there is no doubt in our minds.[5]

EXPERIMENTAL.

Experiment 1. The Effect of Condensed Milk (Rats 3 and 4, Fig. 2).

The diet consisted of cabbage, oats, white bread, fresh vegetables, and condensed milk. The female became pregnant after 48 days and the experiment had to be discontinued. An unusual appetite for condensed milk seemed to be manifested by these animals which suggested to us that a possible increased supply of this form of milk might bring about still better results. The curves compare very favorably with the curves of control rats published by previous investigators and this suggests that careful description of the composition of normal diet is desirable.

Rats 3 (male) and 4 (female).

Days.	Weight. 3.	Weight. 4.
	gm.	*gm.*
0	41.8	35.0
4	60.0	48.7
8	66.9	55.4
12	83.4	68.3
16	95.6	75.9
20	108.7	84.2
24	122.5	93.2
28	134.8	99.4
32	142.5	104.4
36	149.7	116.2
40	146.7	121.4
44	148.7	131.7
48	158.6	154.5

[5] A short time ago a paper was published by my former assistant (Drummond, J. C., *Biochem. J.*, 1916, x, 77) in which he criticizes my results upon chickens stunted by a diet of unpolished rice. In answer I might state that, although his actual figures fully corroborate my statements, his main objection seems to be that even chickens on normal diet do not thrive when kept under laboratory conditions. This fact, of which I am

Experiment 2. The Effect of Yeast Addition to a Normal Diet (Rats 5 and 6, Fig. 2).

The diet consisted of cabbage, oats, white bread, fresh vegetables, condensed milk, and 2 gm. of dried yeast daily. The appearance of these rats as compared with those on artificial diets showed a better growth and a healthier general condition.

Rats 5 (male) and 6 (female).

Days.	Weight. 5.	Weight. 6.
	gm.	*gm.*
0	50.0	49.5
4	69.2	69.8
8	84.5	82.3
12	100.0	92.2
16	107.5	96.4
20	122.6	122.1
24	133.1	123.1
28	146.4	138.2
32	141.3	133.6
36	160.0	150.8
40	172.8	154.3
44	178.5	161.0
48	190.0	168.6
52	199.2	181.1
56	206.1	191.3
60	216.0	196.7
64	217.0	197.8

Experiment 3. The Value for Growth and Maintenance of Oats Alone or with Sodium Bicarbonate.

Rats 29, 30, and 31 received oats alone and Rats 32, 33, and 34 oats with 1 per cent sodium bicarbonate. All the animals showed practically no growth and died. The addition of the alkali has a distinct deleterious effect (Fig. 1).

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fully aware, has been already emphasized in one of our publications. (Funk and Macallum, *Z. physiol. Chem.*, 1914, xcii, 13). Still it also remains a fact that as a result of an inquiry into conditions existing in poultry farms, we are using now a mixture of chicken food, cabbage, and charcoal, the water being entirely replaced by milk, thereby succeeding in diminishing the mortality to a very great extent, in spite of the fact that the birds are kept in small cages. With this improvement we have obtained new data confirming our previous statements on the effect of unpolished rice in arresting the growth of young chickens.

	Rats 29, 30, and 31. Oats alone. Weight (gm.).			Rats 32, 33, and 34. Oats and sodium bicarbonate. Weight (gm.).		
Days.	29.	30.	31.	32.	33.	34.
0	69.6	68.8	68.8	41.6	47.0	56.0
4	70.3	69.7	65.3	41.0	45.6	59.3
8	68.5	58.5	57.8	46.0	42.0	58.3
12	72.6	59.0	61.4	42.0	39.5	55.7
16	75.8	57.7	61.5	34.2		60.4
20	75.5	54.2	59.0			57.6
24	74.4	53.3	55.9			55.0
28	72.8	51.1	52.0			52.1
32	71.4	50.8	51.5			
36	73.6	50.7	52.0			
40	72.8	52.5	50.5			
44	68.5	54.0	44.4			
48	62.2	48.6				
52	63.0	47.0				

Experiment 4. Germinated Oats as Compared with Plain Oats in Their Value for Growth.

These experiments have been performed in analogy to the investigation of Fürst[6] who has found that germinated oats develop antiscorbutic properties (using guinea pigs). It was possible that rats would grow better on germinated seeds, with green parts already developed, but this was not to be the case and strangely enough the animals on oats alone outlived the others. The rats (21, 22, 23, and 24, Fig. 1) received at the beginning germinated oats, which were later supplemented with white bread. Rats 25, 26, 27, and 28 received plain oats and later also white bread. From the 18th day both lots had an artificial diet mixed with 5 per cent plain oats or 10 per cent germinated oats, the latter being found twice as heavy in our case.

[6] Fürst, *V., Z. Hyg. u. Infectionskrankh.*, 1912, lxxii, 121.

	Rats 21, 22, 23, and 24. 0–3 days, germinated oats. 3–18 days, addition of white bread. From the 18th day, Diet 1.				Rats 25, 26, 27, and 28 0–3 days, plain oats. 3–18 days, addition of white bread. From the 18th day, Diet 2.			
	Diet 1.				Diet 2.			
	gm.				gm.			
Casein	22				22			
Sugar	10				10			
Starch	23				28			
Lard	30				30			
Agar	2				2			
Salts	3				3			
Germinated oats	10							
Plain oats					5			
Days.	Weight (gm.).				Weight (gm.).			
	21.	22.	23.	24.	25.	26.	27.	28.
0	39.5	58.0	44.5	33.1	40.3	35.6	36.9	51.0
4	41.3	64.0	48.6	37.3	45.1	40.2	39.7	53.5
8	42.0	51.0	48.2	36.8	44.4	39.8	42.2	54.8
12	39.6	62.2	46.8	34.2	44.8	38.8	41.4	54.6
16	39.4	69.0	49.5	35.0	46.0	40.5	46.3	54.9
20	31.7	56.2	46.5	33.1	48.0	39.5	52.3	56.3
24	31.7	56.2	46.5	33.1	51.9	41.2	53.5	54.3
28			33.4		54.3	37.0	53.2	53.8
32			37.5		54.3	40.4	44.0	51.8
36			37.0		61.4	50.2		56.0
40					65.6	54.4		56.1
44					70.2	58.0		56.0
48					72.0	61.0		55.8
52					72.8	62.2		51.5
56					66.0	55.5		
60					66.5	54.8		

Experiment 5. The Effect of an Addition of Milk to an Artificial Diet.

The animals received the usual artificial diet and in addition to that at the beginning of the experiment 1 cc., later 2 cc. of milk each. The growth of the animals was decidedly slower than on autolyzed yeast. The

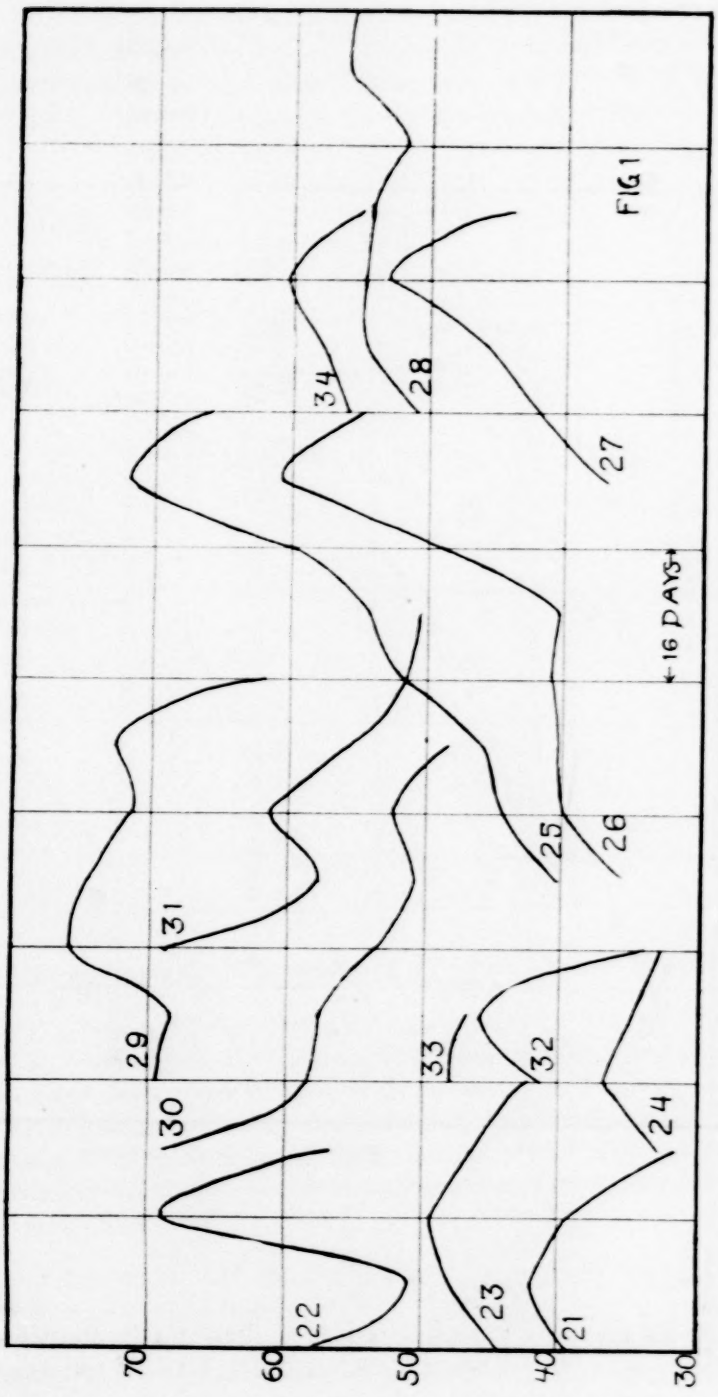

FIG. 1.

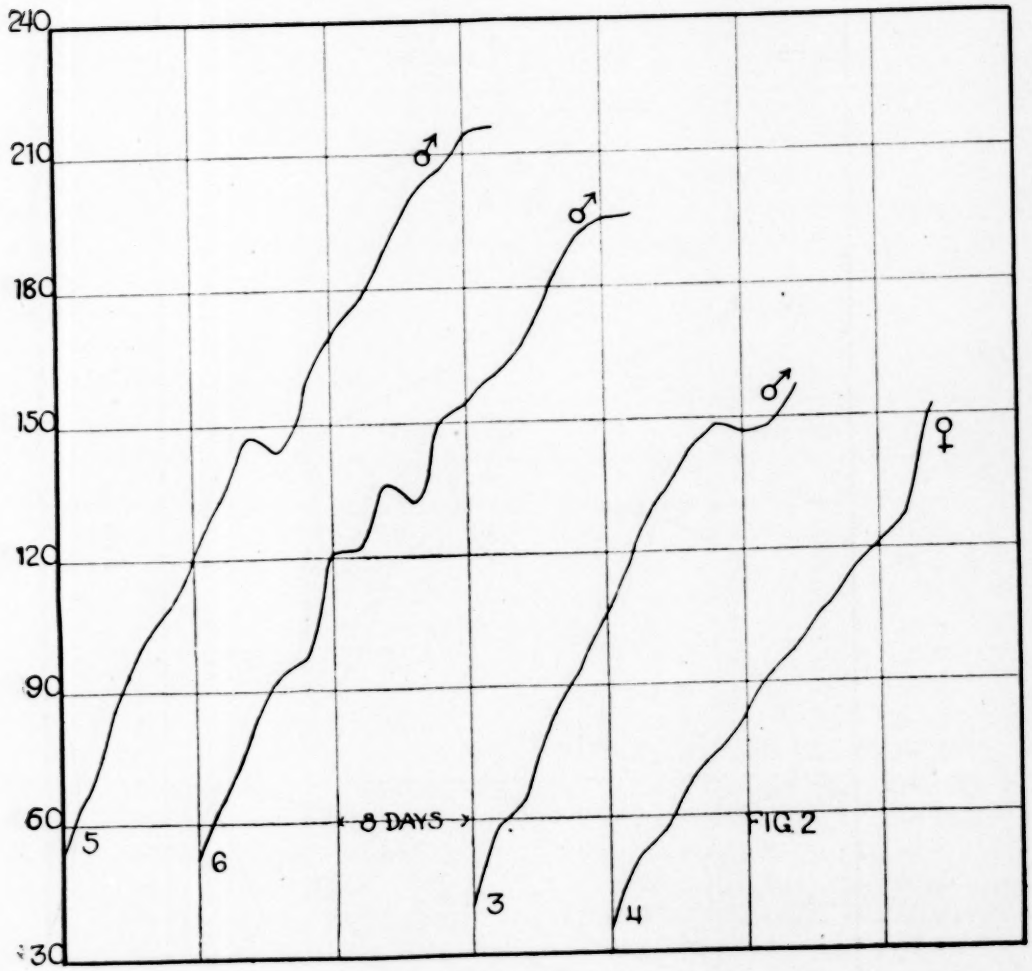

FIG. 2.

table below and the curves for Rats 35 (male) and 36 (female) are represented in Fig. 4.

	gm.
Casein	22
Sugar	10
Starch	33
Lard	30
Agar	2
Salts	3

0–20 days 1 cc. of milk.
20–56 " 2 " " "

Days.	35. Male. Weight.	36. Female. Weight.	Food intake.
	gm.	gm.	gm.
0	33.7	36.6	
4	39.5	41.4	8.5
8	44.1	45.6	7.6
12	49.4	47.4	8.7
16	53.2	47.7	6.3
20	53.7	46.3	6.2
24	52.0	44.8	5.9
28	52.6	45.6	5.6
32	51.5	44.7	5.5
36	59.7	47.8	5.5
40	64.8	47.2	7.5
44	64.0	48.0	5.1
48	63.9	48.7	5.2
52	63.3	51.4	4.4
56	66.0	52.6	3.6

Experiment 6. The Value of Yeast as Protein Supplier.

Casein in our artificial diet was substituted by the same quantity of nitrogen in form of yeast. One male (37) and one female (38) are recorded in this experiment. Rat 37 died with edema after 44 days. As the food intake came gradually down yeast cannot be regarded as a good substitute for protein. There is even a slight indication of a toxic action. Rat 38 which survived 52 days of this experiment has shown an inhibited growth in the last period (Fig. 3).

	gm.		gm.
Yeast	36	Lard	30
Sugar	10	Agar	2
Starch	20	Salts	2

Days.	Weight.		Food intake.
	37. Male.	38. Female.	
	gm.	*gm.*	*gm.*
0	33.3	39.0	
4	45.4	52.3	9.1
8	45.7	65.4	9.8
12	50.3	70.1	10.8
16	56.3	79.2	10.5
20	61.8	86.0	12.4
24	60.1	89.2	11.4
28	58.7	92.2	10.1
32	58.0	97.4	10.5
36	64.0	99.1	10.7
40	65.0	102.0	10.5
44	61.1	103.8	9.8
48		107.6	6.7
52		108.4	5.6

Experiment 7. Action of Orange Juice on Growth.

Rats 53 and 54, males (Fig. 4), were kept on an artificial diet in which the yeast was replaced by orange juice with the result that growth was completely arrested. The animals died after 5 weeks.

	gm.		*gm.*
Casein	22	Agar	2
Sugar	10	Salts	3
Starch	23	Orange juice	10
Lard	30		

Days.	Weight.		Food intake.
	53.	54.	
	gm.	*gm.*	*gm.*
0	28.5	30.0	
4	35.0	35.5	6.6
8	36.0	38.5	5.7
12	37.5	38.5	5.1
16	36.5	37.5	5.6
20	37.5	39.0	5.3
24	38.5	40.0	4.9
28	35.0	36.0	4.6
32	36.0	34.0	4.4
36	31.5		3.0

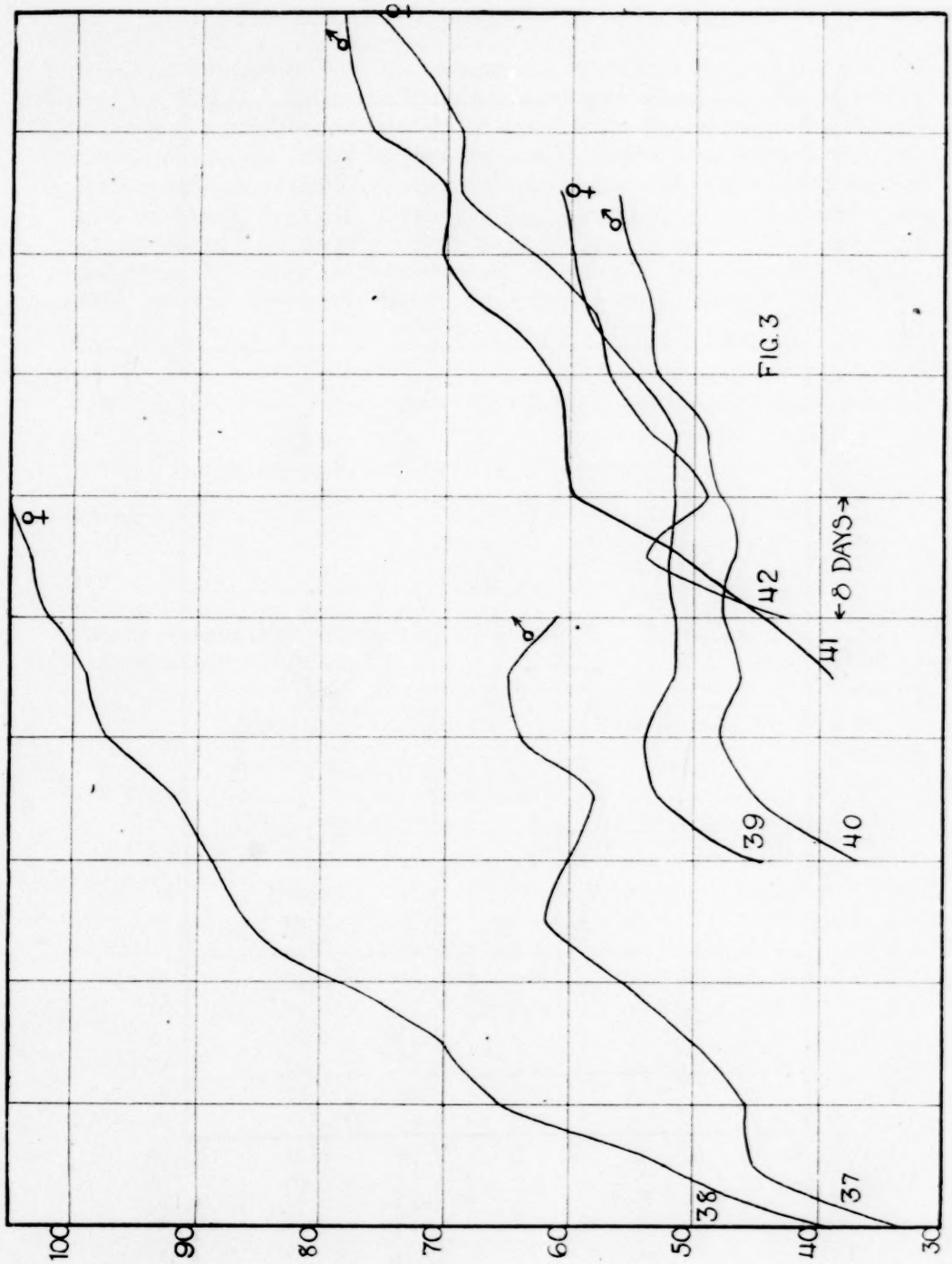

FIG. 3.

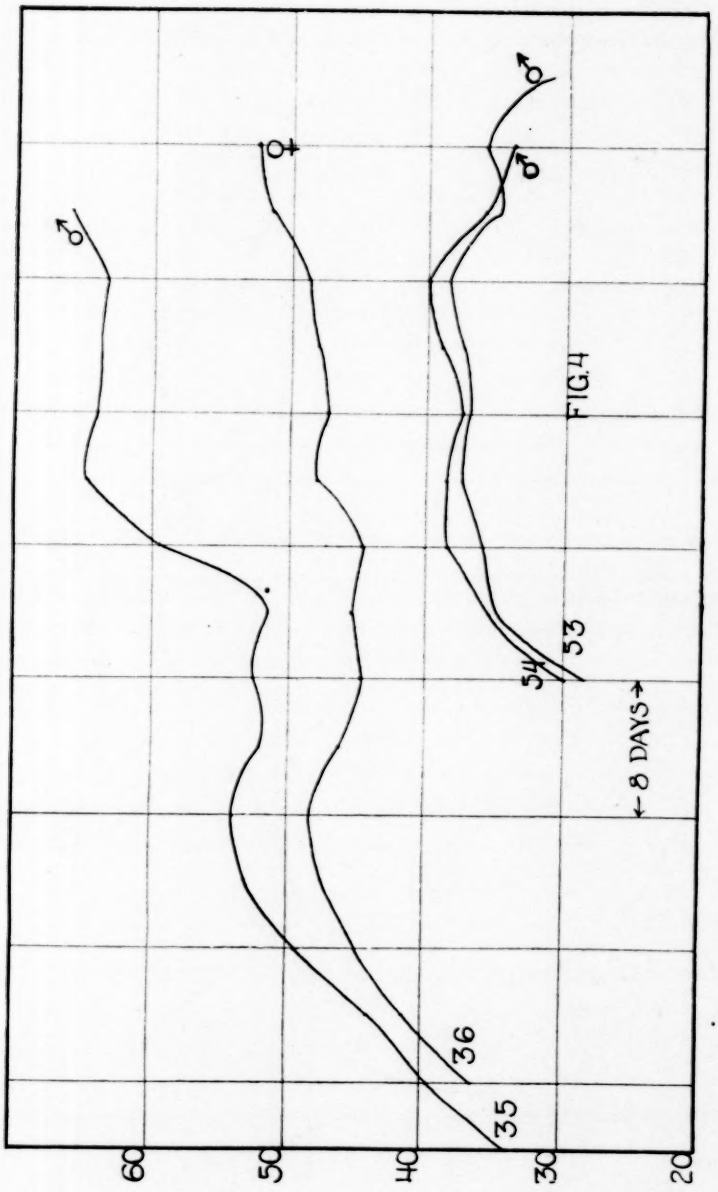

FIG. 4.

Experiment 8. The Value of Lloyd's Reagent as Precipitating Agent for the Growth-Promoting Substance in Yeast.

The precipitation with this reagent was made according to the method used by Seidell. The quantity of the precipitate and the filtrate added to the diet corresponded to 10 cc. of autolyzed yeast which gave good results in our hands. Rats 39 (female) and 40 (male) were kept on the precipitate for 44 days and Rats 41 (male) and 42 (female) (Fig. 3) for the same time on the filtrate. The animals kept on Lloyd's reagent grew decidedly better than those on the filtrate, but the separation of the growth-promoting substance was incomplete. Autolyzed yeast has lost, apparently, most of its antiscorbutic properties by this process.

	Diet 1.	Diet 2.
	gm.	gm.
Casein	22	22
Sugar	10	10
Starch	24	24
Lard	30	30
Agar	2	2
Salts	2	2
Lloyd's precipitate made up to	10 cc.	
Lloyd's filtrate		10 cc.

Days.	Rats 39 and 40, Diet 2.			Rats 41 and 42, Diet 1.		
	Weight.		Food intake.	Weight.		Food intake.
	39. Female.	40. Male.		41. Male.	42. Female.	
	gm.	gm.	gm.	gm.	gm.	gm.
0	44.9	37.0		39.1	43.4	
4	52.8	45.4	9.8	49.5	54.0	11.2
8	54.1	48.0	7.5	55.8	43.7	8.6
12	51.6	46.7	5.8	59.7	51.7	7.9
16	51.6	47.7	7.0	60.5	57.4	8.9
20	51.9	46.9	6.0	61.6	58.0	8.8
24	51.6	47.9	5.5	66.5	63.2	6.3
28	53.6	49.4	6.2	70.2	69.1	9.5
32	55.0	52.5	6.6	70.0	69.0	9.5
36	58.8	52.9	6.2	76.0	72.7	7.5
40	60.0	55.2	5.5	77.8	76.0	9.3
44	61.0	56.3	5.1	78.5	81.2	7.9

SUMMARY.

By an addition of milk or yeast to the normal diet the growth of rats can be accelerated. Oats in dry state or subjected to germination proved to be an inadequate diet for young rats. Yeast cannot substitute the casein in the diet and this very likely because of its toxic properties. Young rats grow less when milk instead of yeast is used in the diet and they do not grow at all on orange juice as addition. Using Lloyd's reagent for precipitation of the growth-promoting substance from autolyzed yeast, it was found that by this process the separation seems to be not complete; furthermore, the yeast loses some of its original value as a stimulant to growth.

COTTONSEED MEAL AS AN INCOMPLETE FOOD.

By C. A. WELLS and P. V. EWING.

(*From the Georgia Experiment Station, Experiment.*)

(Received for publication, July 11, 1916.)

Within the past few years it has been well established that certain injuries to animals, such as beri-beri, result from deficient diets. Thus, purified proteins, carbohydrates, fats, and mineral matter, combined in any proportion, do not produce normal growth. A ration made up of whole wheat, or of certain other cereals, will produce injury through lack of essential food substances. A diet is incomplete if it does not contain an adequate amount of these as yet unidentified accessory substances as well as requisite inorganic salts.

Rommel and Vedder (7) recently advanced the hypothesis, supported by preliminary experiments, that cottonseed meal injury is similar to, if not indeed identical with beri-beri in man. Our own experiments (8) had indicated that in feeding cottonseed meal to pigs to ascertain the injury said to result from this it is necessary to balance the ration, not so much as to the nutritive ratio, determined by the amounts of fats, carbohydrates, and protein present, but rather as to the so called accessory food factors. It is the purpose of the present paper to relate our later experiments bearing upon this phase of the cottonseed meal injury question.

EXPERIMENTAL.

Equipment.—Mated Duroc-Jersey pigs were used. These were 6 weeks old and weighed usually about 6 to 10 kg. each at the beginning of the experiment. They were kept in pens or metabolism cages and fed under conditions previously described in detail (8). We used the pig because it is said to be more susceptible to cottonseed meal injury than are other animals, thus making it easier, presumably, to measure the injury; and when

results are obtained with it there is not the further implied necessity of comparative studies as is always the case where rabbits and similar animals are used. It is inexpensive, and lends itself satisfactorily to metabolism trials in cages.

Feeds.—The feedstuffs were obtained, analyzed, and kept in dry storage throughout the investigation. From the analyses of them, and from the energy values of feeds as given by Armsby and others, the dry matter, digestible protein, and net energy values were calculated on the basis of the feed consumed. The cottonseed meal was of a good grade having a bright yellow color and a sweet odor. The digester tankage was a commercial grade, and the gluten was a good grade of Hoyt's gluten flour. The whole milk was obtained from a Jersey herd and modified by mixing two parts of whole milk with one part of skimmed milk. Preliminary feeding trials were made in each case with a mixture of corn meal 62, wheat middlings 30, and digester tankage 8 parts.

TABLE I.

Dry Matter and Digestible Protein, and Net Energy Values in Therms in 100 Gm. of Air-Dried Substance.

Substance.	Total dry matter.	Nitrogen.	Digestible protein.	Net energy value.
	gm.	*gm.*	*gm.*	*therms*
Corn meal	89.1	1.50	8.4	0.180
Wheat middlings	84.0	2.80	12.8	0.171
Digester tankage	92.7	8.46	47.1	0.161*
Cottonseed meal	91.5	6.66	55.6	0.186
Starch	87.3			0.223
Sugar	100.0			0.256*
Skimmed milk	9.9	0.50	3.6	0.019
Whole (diluted) milk	8.7	0.48	3.4	0.024
Gluten flour	91.3	12.03	63.8	0.180

* Energy values calculated.

In Series 1 a study was made of the comparative effects of large quantities of cottonseed meal, digester tankage, and gluten flour in a limited basal ration; and similarly in Series 2, using cottonseed meal and gluten flour in a basal ration increased to absolute maintenance. In the latter series metabolism trials

were made. The results are summarized in Tables II, III, and IV.

TABLE II.

Series 1. Effects of Large Quantities of Cottonseed Meal, Digester Tankage, and Gluten Flour in a Limited Basal Diet. Daily Averages in Periods.

Date.	Weight.	Feed consumed.	Cotton-seed meal.	Additional water.	Dry matter	Digestible protein	Net energy value.	Injury.	Death.
Pig 1. Diet: Cottonseed meal 25 gm., starch 6, sugar 2.3, skimmed milk 5 cc., per kg. live weight daily.									
1915	*kg.*	*gm.*	*gm.*	*cc.*	*gm*	*gm.*	*therms*		
July 1–10....	10.0	383	250	1,223	305	101	0.64	−	−
" 10–20....	10.5	296	203	1,088	248	82	0.52	+	−
" 20–30....	10.0	223	163	904	200	66	0.42	+	−
Aug. 1–10....	10.0	201	151	1,138	184	61	0.38	+	−
" 10–18....	10.0	148	104	1,050	127	42	0.27	+	−*
Pig 2. Diet: As for Pig 1, with 1.5 gm. gluten flour instead of 5 cc. skimmed milk.									
July 1–10....	10.0	383	250	1,137	314	99	0.66	−	−
" 10–20....	10.0	369	245	1,134	308	97	0.64	+	−
" 20–30....	10.0	333	221	1,116	278	87	0.58	+	−
Aug. 1–10....	11.0	275	190	868	236	75	0.50	+	−
" 10–15....	10.0	120	83	610	104	32	0.22	+	+
Pig 3. Diet: As for Pig 1, with 2.5 gm. digester tankage instead of 5 cc. skimmed milk.									
July 1–10....	10.0	383	250	1,110	320	102	0.67	−	−
" 10–20....	11.0	421	265	1,310	339	108	0.71	+	−
" 20–30....	12.0	311	186	964	238	76	0.50	+	−
Aug. 1– 8....	14.0	224	145	738	185	59	0.40	+	+
Pig 4. Diet: As for Pig 1, with 12.5 gm. gluten flour instead of 25 gm. cottonseed meal.									
July 1–10....	10.0	258		1,304	190	92	0.40	−	−
" 10–20....	10.5	253		1,030	154	75	0.30	+	−
" 20–30....	10.0	202		865	124	60	0.25	+	−
Aug. 1–10....	10.0	187		1,202	115	56	0.24	+	−
" 10–18....	10.0	129		951	79	38	0.17	+	+

* Removed at close of last period after complete injury, to prevent death.

TABLE II—*Concluded.*

Date.	Weight.	Feed consumed.	Cottonseed meal.	Additional water.	Dry matter.	Digestible protein.	Net energy value.	Injury.	Death.
Pig 5. Diet: As for Pig 1, with 18.5 gm. digester tankage instead of 25. gm. cottonseed meal.									
1915	*kg.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*therms*		
July 1–20....	10.0	268		1,252	245	87	0.47	−	−
" 20–Aug. 10....	12.0	294		1,060	267	94	0.56	+	−
Aug. 10–30...	13.0	232		985	210	74	0.45	+	−
" 31–Sept. 20....	13.0	235		983	212	75	0.46	+	−
Sept. 20–Oct. 9.....	13.0	211		1,114	192	69	0.42	+	−
Oct. 9–23	10.0	58		389	53	19	0.12	+	+

DISCUSSION.

It is evident that submaintenance and failure to grow were not due to lack of supply of carbohydrates, fats, protein, and mineral matter. The diets were deficient in some other essential way; so that the value of a diet is not to be measured solely by the amount of protein and net energy it contains.

The lethal period was longer than that previously reported by us (8). This was due, no doubt, to the presence here of additional substances in the diet, such as the small quantity of milk. Dinwiddie (1) found much the same thing in that wheat bran seemed to lessen the injury from cottonseed meal, and the reports of others show that animals do not suffer so much from cottonseed meal if given a variety of food or access to pasture.

Apparently the digester tankage was a more complete food than cottonseed meal or gluten flour. It must have yielded, relatively, a larger proportion of the less abundant valuable food factors, such as tryptophane. Or, in the case of Pigs 1, 2, and 3, the diets may have contained a toxic substance.

The injury was not acute. Through physical appearances and the metabolic characteristics it was manifested long before death. This is not in agreement with the observations of many

TABLE III.

Series 2. Effects of Cottonseed Meal and Gluten Flour when Fed in Low, Medium, and High Maintenance Diets. Daily Averages by Periods.

Date.	Diet.	Feed consumed.	Additional milk.	Additional water.	Total feed consumed.			Weight.	Gain or loss in weight.
					Dry matter.	Digestible protein.	Net energy.		
Pig 6. Experimental diet: Cottonseed meal 3 gm., starch 6, sugar 2.3, per kg. live weight daily, with whole milk as indicated.									
1915-16		*gm.*	*cc.*	*cc.*	*gm.*	*gm.*	*therms*	*kg.*	*gm.*
Dec. 20–23	Preliminary.	236		1,210	203	30.0	0.40	7.5– 7.9	+100
" 24–Jan. 10	Experimental.	80	164	540	82	8.6	0.19	8.2– 6.9	−117
" 11–22	"	80	213	581	83	9.2	0.20	6.1– 6.7	+ 50
" 23–Mar. 20	"	96	630	1,883	86	10.4	0.21	6.7–13.3	+150
Mar. 21–May 5	Recuperative.	720	1,500		769	141.0	1.52	13.3–30.0	+458
Pig 7. Experimental diet: As for Pig 6, with 2.36 gm. digester tankage instead of 3 gm. cottonseed meal.									
Dec. 20–23	Preliminary.	210		1,356	181	27.0	0.36	6.7– 7.2	+120
" 24–Jan. 10	Experimental.	67	126	688	69	6.0	0.16	7.3– 5.5	− 95
Jan. 11–22	"	67	193	856	70	6.5	0.17	5.5– 5.7	+ 17
" 23–Mar. 20	"	78	588	2,166	84	8.5	0.21	5.7–11.2	+ 96
Mar. 21–May 5	Recuperative.	653	1,370		700	122.0	1.38	11.2–28.0	+410
Pig 8. Experimental diet: As for Pig 6, with 1.66 gm. gluten flour instead of 3 gm. cottonseed meal.									
Dec. 20–23	Preliminary.	223		956	192	28.0	0.38	7.1– 7.7	+150
" 24–Jan. 10	Experimental.	67	154	435	69	6.0	0.16	7.7– 6.0	− 94
Jan. 11–22	"	67	210	343	70	6.5	0.17	6.0– 6.2	+ 17
" 23–Mar. 20	"	78	637	1,550	84	8.5	0.21	6.2–12.0	+102
Mar. 21–May 5	Recuperative.	590	1,240		627	112.0	1.25	12.2–30.0	+434

TABLE III—*Concluded.*

Date.	Diet.	Feed consumed.	Additional milk.	Additional water.	Total feed consumed.			Weight.	Gain or loss in weight.
					Dry matter.	Digestible protein.	Net energy.		
Pig 9. Experimental diet: As for Pig 6, with 22.5 gm. cottonseed meal instead of 3 gm. of cottonseed meal. Milk fed separately.									
1915–16		*gm.*	*cc.*	*cc.*	*gm.*	*gm.*	*therms*	*kg.*	*gm.*
Dec. 20–23	Preliminary.	213		493	183	27.0	0.36	6.8–7.2	+100
" 24–Jan. 10	Experimental.	150	144	1,047	137	40.0	0.31	7.2–6.0	− 33
Jan. 11–22	"	120	210	357	111	33.0	0.25	6.0–5.6	− 33
" 23–25	"	54	392	304	55	16.0	0.13	5.6–5.5	− 33
Pig 10. Experimental diet: As for Pig 6, with 12.45 gm. gluten flour instead of 3 gm. cottonseed meal. Milk fed separately.									
Dec. 20–23	Preliminary.	200		1,050	172	26.0	0.34	6.2–6.9	+125
" 24–Jan. 10	Experimental.	125	134	653	114	33.0	0.25	6.9–5.9	− 55
Jan. 11–12	"	125	206	730	115	34.0	0.26	5.9–6.9	− 83
" 13–24	"	25	483	580	29	10.0	0.07	6.9–5.5	−120
Pig 11. Experimental diet: As for Pig 9, with 70 cc. whole milk per kg. live weight daily, fed separately from beginning of experimental period.									
Jan. 22–25	Preliminary.	330		600	284	42.0	0.56	9.9–10.0	+ 25
" 26–Apr. 26	Experimental.	105	875	865	102	30.0	0.23	10.0–15.1	+ 55
Apr. 27–May 11	Recuperative.	503	1,057		536	137.0	1.05	15.1–25.5	+700
Pig 12. Experimental diet: As for Pig 11, with 12.4 gm. gluten flour instead of 22.5 gm. cottonseed meal.									
Feb. 5–8	Preliminary.	396		800	340	50.0	0.68	11.9–12.2	+ 75
" 9–Apr. 30	Experimental.	468	1,302	4,616	417	124.0	0.95	12.2–25.0	+158
May 1–25	Recuperative.	1,080	2,270		1,256	185.0	2.23	25.0–40.0	+600

TABLE IV.

Series 2. Results of Metabolism Trials with Pigs 6, 9, 10, 11, and 12. Daily (24 Hour) Averages of 3-Day Periods.

Date.	Pig.	Weight.	Diet.	Urine.								Feces.		Nitrogen balance.	Nitrogen utilization.
				Volume.	Acidity. Log.	Ammonia nitrogen.	Urea nitrogen.	Total nitrogen.	Creatinine.	Sulfur oxidized.	Total sulfur.	Weight.	Nitrogen.		
1915-16		*kg.*		*cc.*		*gm.*	*gm.*	*gm.*	*gm.*			*gm.*	*gm.*		*per cent*
Dec. 20–23..	6	7.1	Preliminary, 236 gm.	154	7.4	0.284	1.161	1.804	0.184	0.101	0.140	49	1.112	+2.748	80
Apr. 18–20..	6	17.0	Recuperative, 567 gm. with 1,200 cc. milk.	963	7.0	2.141	2.874	6.631	0.753	0.438	0.575	567	7.964	+5.113	76
Dec. 20–23..	9	6.4	Preliminary, 213 gm.	132	6.2	0.102	0.588	1.365	0.173	0.075	0.115	65	1.248	+2.499	76
Jan. 10–12..	9	7.2	Experimental. Ate 144 gm. cottonseed meal with 144 cc. milk.	225	7.2	0.126	2.059	2.174	0.071	0.107	0.136	57	1.532	+6.504	85
Dec. 20–23..	10	6.0	Preliminary, 200 gm.	195	7.1	0.281	1.152	1.710	0.220	0.134	0.185	56	1.220	+1.870	76
Jan. 10–12..	10	7.7	Experimental. Ate 95 gm. gluten flour with 138 cc. milk.	330	6.9	0.504	6.237	6.930	0.116	0.285	0.343	12	0.466	+4.694	96
Dec. 20–23..	11	6.3	Preliminary, 210 gm.	245	7.4	0.240	1.132	1.739	0.178	0.112	0.139	108	1.248	+2.125	75
Apr. 13–15..	11	13.6	Experimental. Ate 124 gm. cottonseed meal with 952 cc. milk.	1,289	7.2	1.348	7.368	13.529	0.299	0.508	0.845	61	1.265	−1.776	90
May 9–11...	11	25.5	Recuperative, 850 gm.	1,330	7.0	2.088	4.223	8.472	0.891			485	7.265	+5.663	64
Dec. 20–23..	12	6.6	Preliminary, 220 gm.	185	6.5	0.320	1.147	1.584	0.170	0.189	0.231	92	1.180	+2.392	76
Apr. 13–15..	12	25.0	Experimental. Ate 311 gm. gluten flour with 1,750 cc. milk.	4,600	5.7	2.087	21.771	34.650	0.263	1.482	2.382	13	0.663	+10.819	98

other investigators. We are inclined to believe, however, that in the reported cases of acute cottonseed meal injury, careful periodic measurements would have disclosed the injury before death. This is important; for if well nourished animals die of the injury as often claimed, the same cannot be due to a deficient diet and inanition, but must be due to a toxic effect.

The ingestion of smaller quantities of nitrogenous foods as the injury advanced, and the retarding effect of additional nitrogenous foods, indicate that excess of nitrogen did not cause the injury. When upon an assured maintenance diet the sugar, starch, and other foods did not produce injury. And since the pigs, upon full maintenance, would not eat much cottonseed meal, it was necessary to feed the basal diet separately that it might all be eaten.

Postmortem examinations were made in all cases, but the results are omitted here, because, as shown in detail in a previous publication (8), they varied so widely as to have little interpretive value. Among those characteristics which were found most constant may be mentioned: a rough coat; dry, harsh, and scaly skin; weakness and emaciation as shown by physical appearance and entire absence of fat from kidneys and mesentery; and bones usually though not always brittle.

From results in Series 2 it appears that injury was not prevented by that amount of milk which Hopkins (3) found sufficient for protection of rats receiving a deficient diet. Even Dietrich's maintenance allowance for pigs was inadequate here. But *absolute maintenance was assured when the milk was increased to 70 cc. per kg. live weight daily.*

The death of Pig 10 was due probably to a tumor-like internal injury revealed by postmortem examination. In all other cases death resulted in part from lack of sustenance. It should be noted, however, that Pig 9 died 4 days after its maintenance was made absolute by the addition of milk. The fact that it did not respond, as did Pigs 6, 7, and 8, to this increased milk allowance, leaves the possibility to be reckoned with that the cottonseed meal contained a specific injurious effect apart from its lack of essential food factors.

The metabolism trials confirm the results formerly reported (8) to the effect that on such diets the pig is unable to deflect nitrogen from urea to form ammonia to neutralize any excess acids present. The low utilization of nitrogen in diets highly efficient for growth and its high utilization in diets inefficient for growth may be explained on the basis that the animals when on restricted diets utilized the nitrogen to the greatest possible extent in an effort to obtain those particular substances much needed yet present to a limited extent in the diet. This explanation, of course, is not in keeping with the commonly accepted view that the character of a ration does not affect the degree of utilization of any food constituent. Yet it should be borne in mind that Mendel and Fine (4, 5, 6) have shown that the proteins of corn, wheat, and meat are utilized in carnivora to approximately the same extent; namely, ± 93 per cent. One would expect, therefore, upon mixing these substances together, as was essentially the case in our preliminary and recuperative diets, to obtain an equivalent utilization of the nitrogen. But this did not happen. The nitrogen was utilized to approximately 76 per cent, which is about the figure given in the literature for this food mixture. Constant watch was kept to see that the pigs did not eat the feces; and from the results in Table IV it is evident that in the restricted diets anabolism was at its maximum, while in the case of the normal preliminary and recuperative diets much of the nitrogen went to waste. When on a bountiful diet, the animal apparently selected with ease those substances demanded by the specifically established physiological processes of the body, including nitrogen, letting much go to waste through the feces; but when the diet was restricted, the tendency was to increase the nitrogen utilization beyond that required by the body in an effort to obtain the other necessary substances. A reference to our related work on the associative action of foods with steers (2) where cottonseed meal was fed alone as a restricted diet, and with corn silage making a fairly normal diet, shows this same increased utilization of nitrogen on the restricted diet. The results, of course, are based upon limited data and are indicative rather than conclusive.

The decrease in creatinine in the urine when the pigs were changed from the normal to the experimental diets was not associated with the possibility of preformed creatinine in the digester tankage fraction, as shown by separate trials not recorded here. The creatinine decrease indicated a condition of inanition and impaired muscular activity. The creatinine not eliminated was utilized, perhaps, as food, for which there was an increased demand in the one-sided diets. Throughout it is evident that inanition played an important part in the injury.

The results show that in studies of so called cottonseed meal injury with pigs it is necessary to eliminate the interference of inanition by feeding a basal diet which contains those substances requisite for normal growth in addition to the dry matter, digestible protein, and net energy usually considered. The diet must contain milk or some combination of other foods of known growth-producing value.

CONCLUSIONS.

1. Cottonseed meal is an incomplete food. This is true even when it is fed with sugar and starch to a wide nutritive ratio.

2. Pigs upon an absolute maintenance diet ate in addition only small quantities of cottonseed meal and were not greatly injured by it.

3. So called cottonseed meal injury is due in large part to inadequate diets.

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THE PHYSIOLOGICAL RELATION OF PLANT CAROTINOIDS TO THE CAROTINOIDS OF THE COW, HORSE, SHEEP, GOAT, PIG, AND HEN.

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It has been shown by the author[1] that the natural yellow pigments which characterize the milk fat and tissue fat of the cow, the milk fat of the human, and the egg yolk and tissue fat of the hen, owe their origin to the carotin and xanthophyll pigments present in the food. These natural plant pigments, which are collectively known as carotinoids, appear to be transferred in some simple fashion by the blood serum from the digestive tract to the fat-forming organs where they became incorporated with the synthesized fat. Just how this is brought about is at present obscure. The author has shown conclusively, however, that the presence of pigment in deposited tissue fat, or in the egg yolk, or in the milk fat depends exclusively upon the presence of the same pigment in the blood serum. For example, when cows were fed rations nearly devoid of carotinoids, these pigments practically disappeared from the blood serum, and the milk fat became almost colorless. Similarly, when laying hens were fed rations nearly devoid of carotinoids the blood serum, as well as the egg yolks, showed almost complete absence of these pigments.

Probably the most interesting result of the study of the yellow pigments which characterize animal fat has been the demonstration of the fact that the yellow pigments which are resorbed from the digestive tract of the cow consist almost exclusively of carotin, very small portions of the xanthophyll group of pigments being present. The pigment which characterizes the

[1] Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191. Palmer, L. S., *ibid.*, 1915, xxiii, 261.

egg yolk and tissue fat of the hen, on the other hand, consists almost exclusively of xanthophyll, relatively little carotin being resorbed. The pigments of human milk fat, in the case of one woman studied by the author, appear to consist of much more nearly equal parts of the two classes of carotinoids.

Another interesting fact developed with reference to the manner in which the blood serum of the cow and hen carries the pigment characteristic of the species. In the case of the cow the carotin of the blood serum is in some sort of physical or chemical combination with albumin. This compound, which the author has called caroto-albumin, is easily isolated from fresh serum. It is readily soluble in water, while carotin itself is soluble only in the fat solvents. In the case of the hen serum, however, no such combination appears to exist between xanthophyll and protein, the pigment being readily extracted from the serum with ether, which is in marked contrast to the complete failure of pure ether to extract any carotin from the fresh blood serum of the cow.

Carotinoids in Other Species of Animals.

The presence of carotin in the cow and xanthophyll in the hen, in each case to the relative exclusion of the other members of the carotinoid group, raised the question of what pigments characterize the tissue fat and blood serum of animals of other species. The author has recently had an opportunity to investigate this question in the case of the horse, pig, sheep, and goat. It is the purpose of this paper to set forth briefly the results of that study.

Carotinoids in Swine.—Considerable interest was attached to the study in the case of the swine, and also in the case of the sheep, reported below, on account of the fact that the tissue fat of each of these species is characteristically colorless. The question involved was whether this would be found to be due to differences between these species and the cow and hen at the seat of the fat synthesis, or to the failure of the carotinoids to be resorbed from the digestive tract.

Blood was secured from three pure-bred swine, representing the Duroc-Jersey, Poland China, and Berkshire breeds. For several weeks before they were slaughtered the animals had run

on pasture containing an abundance of fresh blue-grass. The conditions were thus most favorable for the largest transfer of carotinoids into the blood stream. The blood from each of the animals was defibrinated at once, and the plasma centrifugalized. The serum was tested for the presence of carotin and xanthophyll by several methods. The reader is referred to the previous work by the author[2] for a description of these methods. Desiccation of the serum with plaster of Paris and extraction with petroleum ether and ether after moistening with absolute alcohol is the easiest and most reliable method for extracting the carotinoids from blood serum.

The results in the case of each of the three pigs were absolutely negative. Not a trace of either carotin or xanthophyll was present in the blood serum.

Carotinoids in Sheep.—Blood was secured from five pure-bred ewes, representing the following breeds: Dorset, Hampshire, Merino, Shropshire, and Southdown. Each of the ewes was in milk with the exception of the Hampshire. The animals received an abundance of fresh blue-grass pasture for several weeks before they were bled. The blood was secured from the jugular vein by means of a trocar, about 150 cc. being obtained from each animal. The four ewes in milk were milked at the same time the blood was drawn, the milk from the Dorset and Southdown ewes being combined, as was also that from the Merino and Shropshire ewes.

The blood from each animal was defibrinated, the plasma centrifugalized, and the serum examined for the presence of carotinoids. The extracts from 10 cc. portions of the serum failed to reveal any pigment whatever, but the combined extracts from about 150 cc. of serum, when concentrated to a volume of 5 to 10 cc. showed a pale yellow color, which was completely extracted from 85 per cent alcohol by both petroleum ether and carbon disulfide, indicating that the pigment was carotin. The merest traces of pigment thus appeared to be present in the blood of the five sheep examined.

The milk obtained from the ewes was churned, without separating, on account of the small amount obtained. The butter in each case was "dead" white in color, but when rendered it

[2] Palmer and Eckles, *J. Biol. Chem.*, 1914, xvii, 223–236.

had a faint greenish tint which may have been due to the presence of carotin. Not enough butter fat was secured to investigate this point further.

Carotinoids in the Goat.—Blood was secured from a she-goat of the Angora breed while receiving an abundance of fresh green feed. The goat was dry at the time, so that no milk was obtained.

The study of the blood serum indicated the possible presence of faint traces of carotin. The extract of 10 cc. of serum would have been judged colorless, however, had it not been compared carefully with a similar amount of fresh petroleum ether.

The study of the yellow pigmentation in the case of the goat was of special interest owing to the investigation of the transference of pigments into milk by Dombrowsky,[3] who fed a goat on carrots and observed whether the carotin passed into the milk. Two feeding trials 2 weeks apart resulted in the observation by Dombrowsky that the milk was yellowish on the evening of the 2nd day of the first feeding trial and was clearly yellowish on the 3rd and 4th days of the second trial. An attempt to show that the color was due to carotin resulted in failure in each case. The test used was the application of the well known color reaction of carotin with concentrated H_2SO_4, to the residue from an ether extract of the milk. If considerable carotin was present, which the author doubts, the test used by Dombrowsky would have been vitiated by the fat present in the extract, since no attempt was made to eliminate it. It is unfortunate that Dombrowsky failed to observe whether the fat in the milk showed an increased color accompanying the carrot feeding. A statement of the breed of the goat used would also have enhanced the value of his results.

Carotinoids in the Horse.—Blood serum was secured in the usual way from a horse which had been running on fresh bluegrass pasture for several weeks. The serum was found to be rich in carotin. No xanthophyll was found to be present. The manner in which the horse serum carries the carotin was found to be identical with that of the cow. The isolation of the water-soluble caroto-albumin from 100 cc. of serum was carried out with the greatest ease. The method used may be of interest to the reader and will be described briefly.

[3] Dombrowsky, *Arch. Hyg.*, 1904, l, 183.

100 cc. of the fresh serum were diluted with an equal volume of water and treated with 200 cc. of saturated $(NH_4)_2SO_4$ solution. After standing 1 hour the globulin was filtered off on a Buchner, using suction. The deep yellow filtrate was heated carefully in a water bath to 79°C. The heavy coagulum which formed was filtered off on a Buchner, using suction. The golden yellow filtrate was treated with $(NH_4)_2SO_4$ in substance until precipitation of the pigmented protein occurred. This was filtered off, using suction, which was continued until the precipitate on the funnel was free from visible moisture. It had a dark yellowish brown appearance. It dissolved readily in 50 cc. of warm water, giving a deep yellow solution. It was not possible to extract the carotin from this solution until the pigment was first freed from the protein with alcohol.

The amount of carotin carried by a unit volume of horse serum was found to be considerably less than that carried by the serum of the cow. Where 5 cc. of horse serum gave an extract giving a reading of 3.75 units of yellow and 0.4 units of red in the Lovibond tintometer in a 1 inch layer and 12.5 cc. volume, a similar amount of cow serum under like conditions gave an extract in former studies showing as high as 27.0 units of yellow and 0.9 units of red.

No opportunity was offered to investigate the yellow pigment which characterizes the tissue fat of the horse, but it may with safety be assumed to be carotin in view of the results of the studies made by the author with the cow and hen.

CONCLUSIONS.

A definite physiological relation exists in all species of animals between the pigmentation of tissue fat with carotinoids and the presence of these pigments in the blood serum. Species whose tissue fat is colored with the carotinoids, which include the cow, horse, and hen, carry the pigments in the blood serum. Species, the tissue fat of which is characterized by being colorless, including swine, sheep, and goats, carry only insignificant traces of the carotinoids in the blood serum even under the most favorable conditions.

An abundance of carotin in the blood serum and in the tissue

fat is not a specific characteristic of the ruminants, as indicated by the absence of carotin from the blood serum and tissue fat of sheep and goats and its presence in the horse. The factors underlying the difference between these species in this respect offer an interesting field for study, as do those factors which cause the relatively exclusive resorption of carotin in the case of the cow and horse, and xanthophyll in the case of the hen.

THE RELATION OF THE UNIDENTIFIED DIETARY FACTORS, THE FAT-SOLUBLE *A*, AND WATER-SOLUBLE *B*, OF THE DIET TO THE GROWTH-PROMOTING PROPERTIES OF MILK.*

By E. V. McCOLLUM, N. SIMMONDS, and W. PITZ.

(From the Laboratory of Agricultural Chemistry of the Wisconsin Experiment Station, Madison.)

(Received for publication, July 31, 1916.)

The results of nearly a thousand feeding experiments which we have conducted with our rat colony during the last few years all support the working hypothesis which we have explained and supported by experimental evidence in a number of previous papers.[1] This assumes the necessity of two dietary essentials as yet unidentified in the diet for growth or prolonged maintenance; *viz.*, the fat-soluble A of butter fat and certain other foodstuffs, and the water-soluble B which we have supplied by means of extracts prepared with water or alcohol from several foodstuffs.

These substances, or possibly groups of substances, which are indispensable from the diet during growth are of interest in relation to their possible formation within the maternal organism in connection with milk secretion. Experimental inquiry has not yet revealed the degree of dependence of animals upon a supply of these two dietary factors after growth has been completed. The evidence available would seem to point to the necessity of at least one of them, the water-soluble B, throughout life, for extracts which contain it induce relief of polyneuritis, and this may have its incidence at any time during life. The

* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181, 231. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916, xxv, 105. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239.

failure of rats to remain in a state of health on diets lacking the fat-soluble A we have pointed out in the papers cited.

The possibility still remains that the gonads, particularly in the female, or the mammary tissue, may be capable of producing one or both of these two dietary factors for the preservation of the young. The prevalence of infantile beri-beri among children whose mothers' diet is insufficient to prevent this disease may be raised against this assumption. The possibility still remains, however, that the diet of these people, which is restricted as to source and variety, and is of poor quality, may be inadequate with respect to the preventive substance itself and also as to its content of a possible precursor from which it might be formed through the peculiar property of a special organ in a manner analogous to the formation of the various hormones of internal origin.

In order to test this point it seemed desirable to observe the ability of normal female rats to suckle their young when confined to a diet which is known to be adequate for growth when satisfactory amounts of the unidentified A and B are added, and inadequate for growth when either of these factors is omitted. The paucity of such a diet in possible precursors is hardly to be expected and the results should enable us to decide whether these two essential substances pass into the milk only when they are furnished in the diet.

Method of Procedure.

Female rats were fed a ration of natural foodstuffs until they delivered their young. When the birth of young was observed the litter was in all cases reduced to four, in order that the nutritive undertaking for the mother in rearing them might not be excessive. Several series of experiments were conducted in which the basal ration consisted of purified casein, dextrin, inorganic salts, and agar-agar. It was adequate for growth except for the absence of the dietary factors A and B. In a second series the same ration plus butter fat (fat-soluble A) was employed. In a third series the water-soluble B only was added to the basal ration in the form of an alcoholic extract of wheat embryo, and in a fourth series both the fat-soluble A and the water-soluble

B were added. The results of feeding these rations to nursing mothers, as shown by their ability to induce growth in their young, are shown in Charts 3 to 6. For comparison we include also the records of rats fed a ration during the lactation which contained both the unidentified A and B but lacked protein.

DISCUSSION OF RESULTS.

The observation of Decaisne,[2] during the siege of Paris, that even during fasting or partial fasting vigorous young women were able to keep up a flow of milk sufficient to induce some growth in their infants, is in harmony with unpublished data obtained by Professor Hart of this laboratory. His data show that cows tend to keep up the flow of milk when the character of the diet is such as to induce a decided negative nitrogen balance, and that the product closely approximates the normal composition with respect to the constituents usually determined in analysis. This supports the view that the failure of the young to grow in certain of these experiments was not due to a depression of the milk supply, but to the production of milk which was not adequate for the promotion of growth on account of its deficiency in one or both of the unidentified factors we are considering.

It seems necessary to conclude that these two constituents of the diet pass into the milk only as they are present in the diet of the mother, and that milks may vary in their growth-promoting power when the diets of the lactating animals differ widely in their satisfactoriness for the growth of young.

It seems further justifiable to conclude that the chemical natures of the fat-soluble A and the water-soluble B, whether these represent in each case a single substance or a group of substances, are of such a character that they cannot be formed within the animal body from any of the cleavage products of proteins.

[2] Decaisne, E., *Gaz. méd. Paris*, 1871, 317.

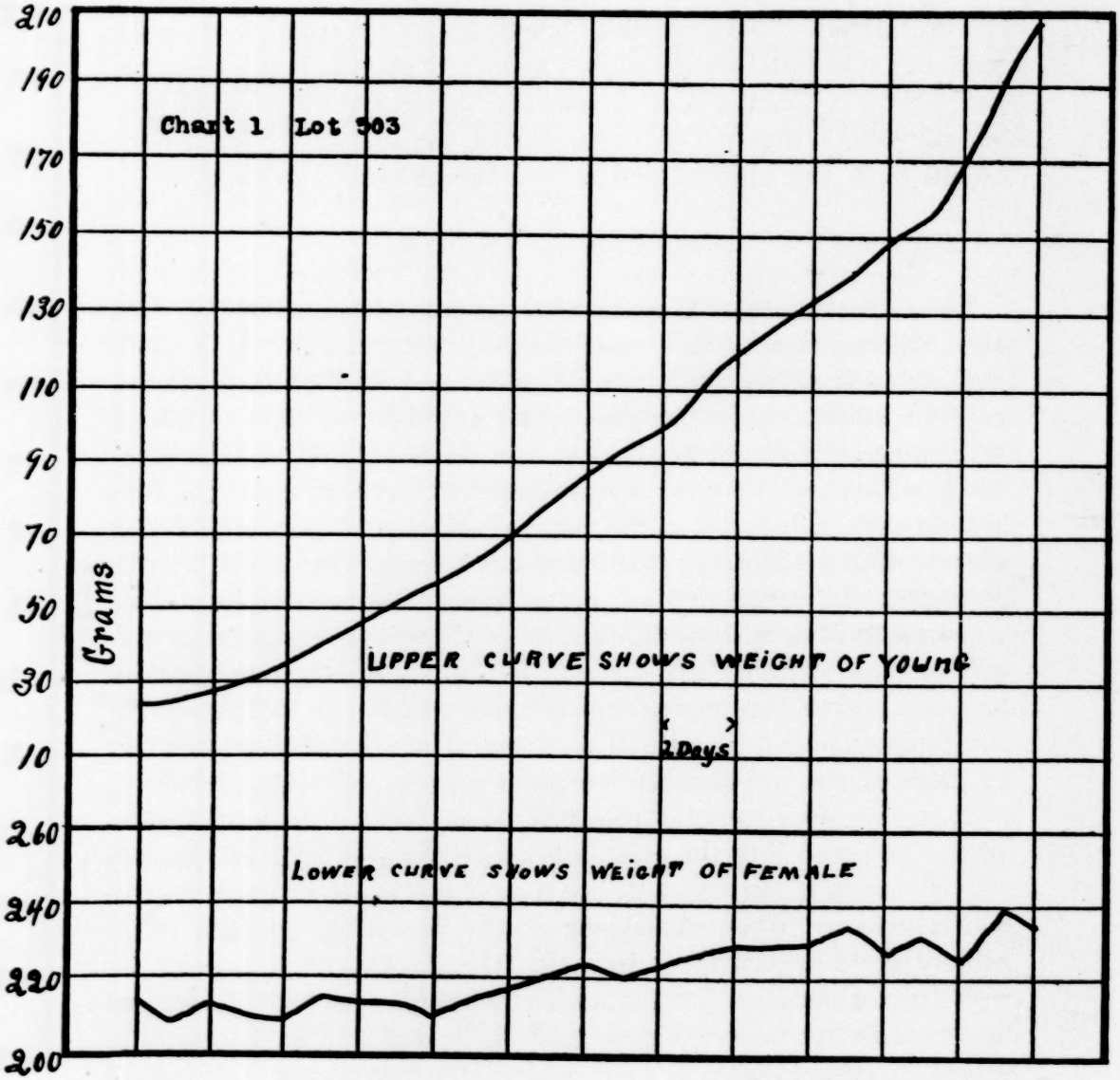

CHART 1.

Chart 1, Lot 503, illustrates the rate of gain of a litter of four young rats when the ration of the mother consisted of a highly satisfactory mixture of natural foodstuffs. The mother was able to increase her weight while the young were gaining at a remarkable rate.

We have described elsewhere the growth and reproduction records of rats confined to this diet.[3]

[3] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 638.

The ration of this rat consisted of:

Casein	10.0
Milk powder	10.0
Wheat	64.0
Salt mixture (211)	3.6
Dextrin	7.4
Butter fat	5.0

Composition of Salt Mixture 211.	
NaCl	0.520
$CaH_4(PO_4)_2H_2O$	0.276
Ca lactate	1.971
K citrate	0.799
Fe acetate	0.100

Chart 2, Lot 640, Period 1, illustrates the extent to which the mother can sacrifice her own tissues for the preservation of the young when her diet contains all the essential factors except protein, and shows the rapid loss in weight of the mother and the slow growth of the young. Upon the addition of 18 per cent of casein in Period 2 after 5 days and 9 days respectively on the protein-free diet, they were able to increase perceptibly the rate of growth of the young and practically began to maintain their own body weights.

The ration in Period 1 (Lot 640) consisted of:

Dextrin	89.3
Salt mixture (185)	3.7
Butter fat	5.0
Agar-agar	2.0

Composition of Salt Mixture 185.	
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2O$	0.540
Ca lactate	1.300

The dextrin carried the alcoholic extract of 10 gm. of wheat embryo.

In Period 2, 18 per cent of casein replaced an equivalent amount of dextrin.

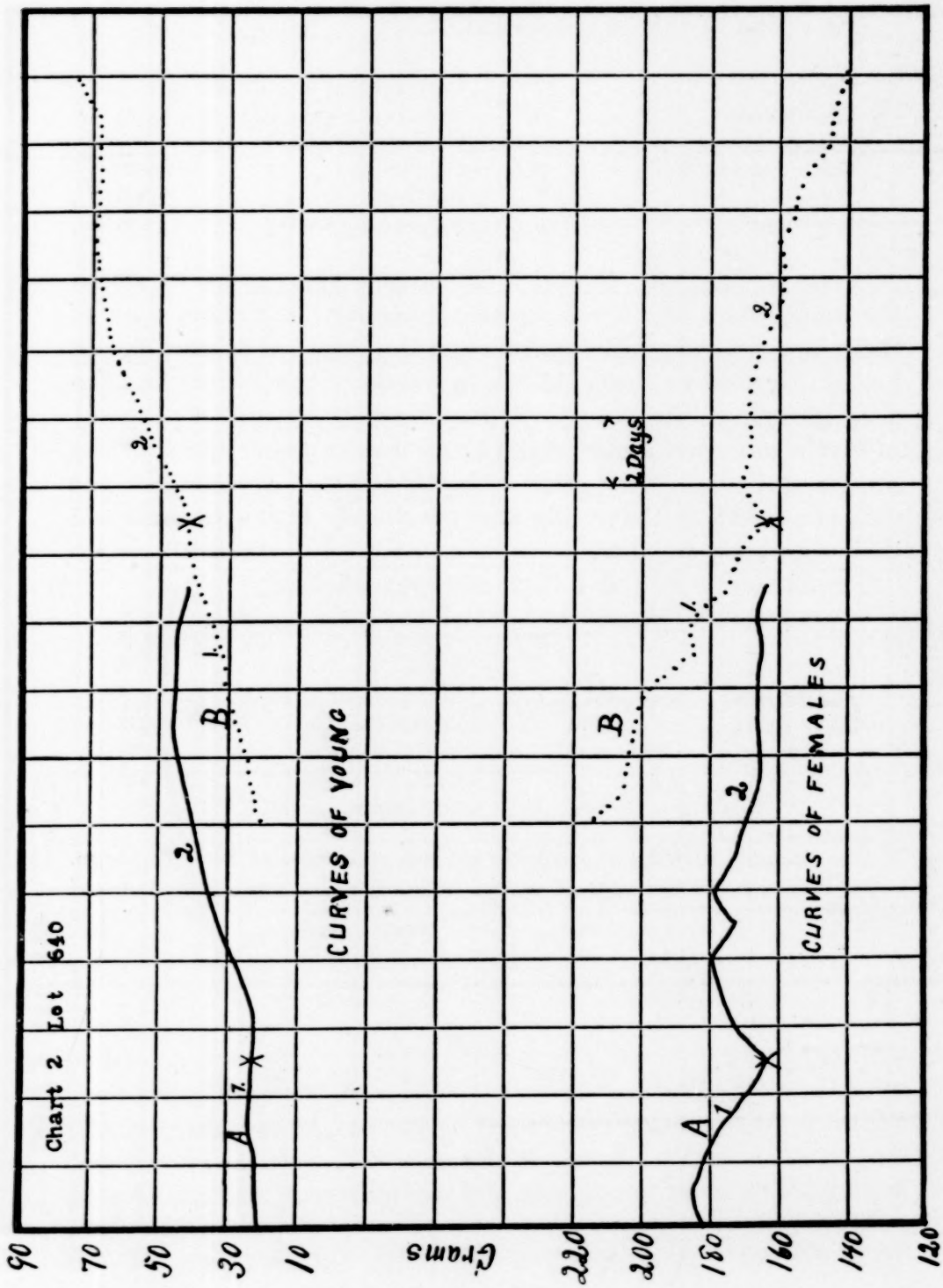
Chart 2 Lot 640
CURVES OF YOUNG
CURVES OF FEMALES
Grams
2 Days
A
B
1
2
90
70
50
30
10
220
200
180
160
140
120

CHART 2.

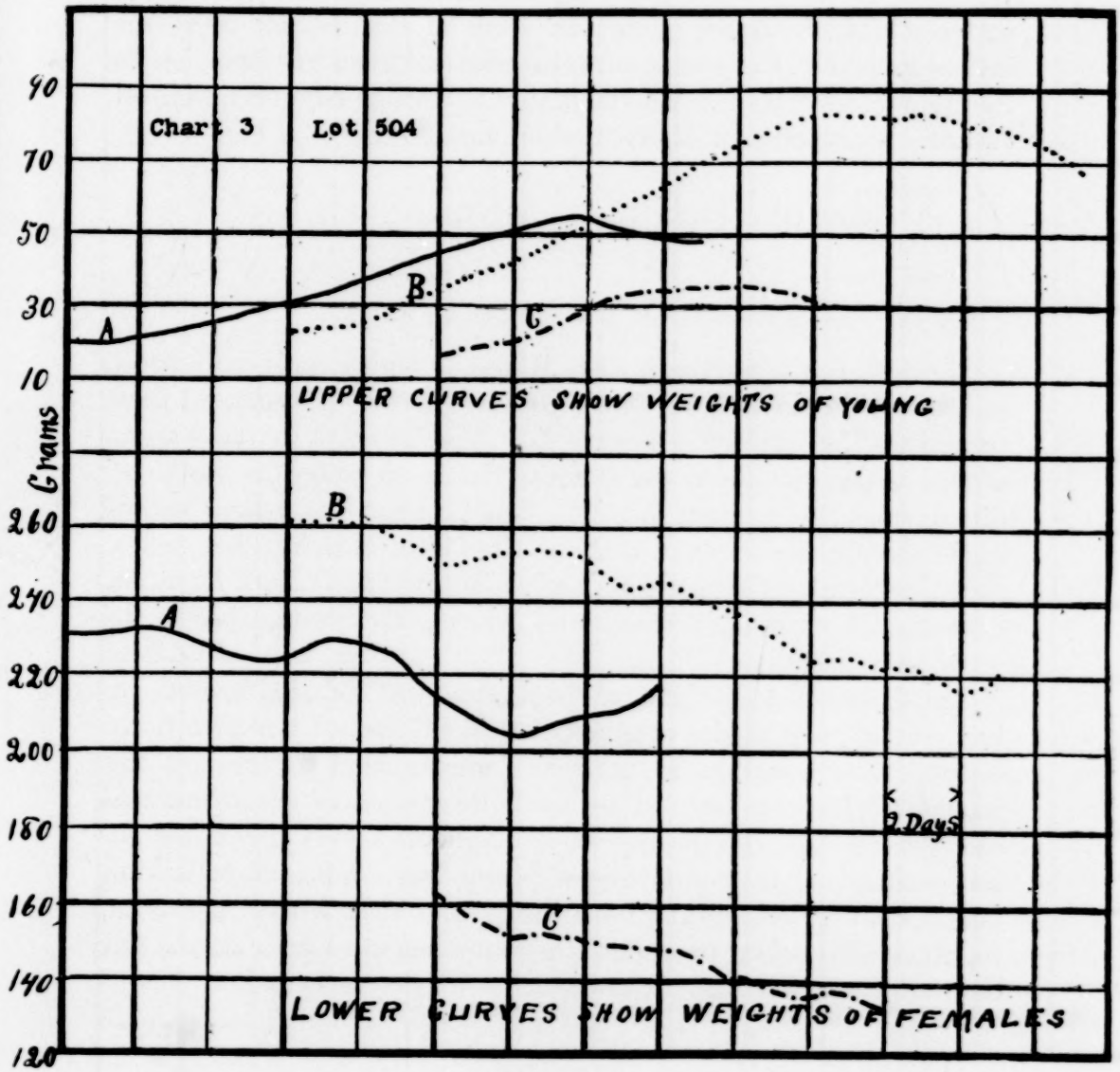
Chart 3
Lot 504
UPPER CURVES SHOW WEIGHTS OF YOUNG
LOWER CURVES SHOW WEIGHTS OF FEMALES
A
B
C
Grams
40
70
50
30
10
260
240
220
200
180
160
140
120
9 Days

CHART 3.

Chart 3, Lot 504, illustrates the degree to which the mother can induce growth in the young when the diet contains an adequate amount of protein, carbohydrate, and inorganic salts. Without the unidentified dietary factors, the fat-soluble A and water-soluble B in the food, the milk of the mother does not induce growth in the young except as she sacrifices her own tissues for their preservation. She is unable to do this beyond a limited extent. Composition of the ration, Lot 504:

Casein	18.0
Salt mixture (185)	3.7
Dextrin	76.3
Agar-agar	2.0

For the composition of Salt Mixture 185 see above.

Chart 4, Lot 506, shows Rats B and D which received a food mixture which lacked only an alcoholic extract of a natural food to supply the unknown dietary B. The increase in the weight of the young was about the same as that seen when the mothers' diet lacked the protein factor. Rat A kept her young growing slowly during 16 days without the factor B in her diet, when growth ceased. On changing her to Ration 503 (Chart 1) which contained both milk powder and wheat, the young began to grow very rapidly.

Rat C was not so vigorous an animal and induced growth in her young only at the expense of decided loss of her own body tissue. The addition of both the unidentified factors by the inclusion of butter fat and an alcoholic extract of wheat embryo did not improve the quality of her milk to a very great degree, but checked the rate of decline of the mother. When given Ration 503 (Chart 1) containing both the A and B in liberal amounts, together with better proteins, the young began to grow on the 3rd day after the change. Composition of the ration, Lot 506:

Casein	18.0
Dextrin	71.3
Salt mixture (185)	3.7
Butter fat	5.0
Agar-agar	2.0

For the composition of Salt Mixture 185, see above. Period 2, Rat A was given Ration 503. See Chart 1. Rat C. The dextrin of her ration carried the alcoholic extract of 10 gm. of wheat embryo. Period 3, Rat C was given Ration 503. See Chart 1.

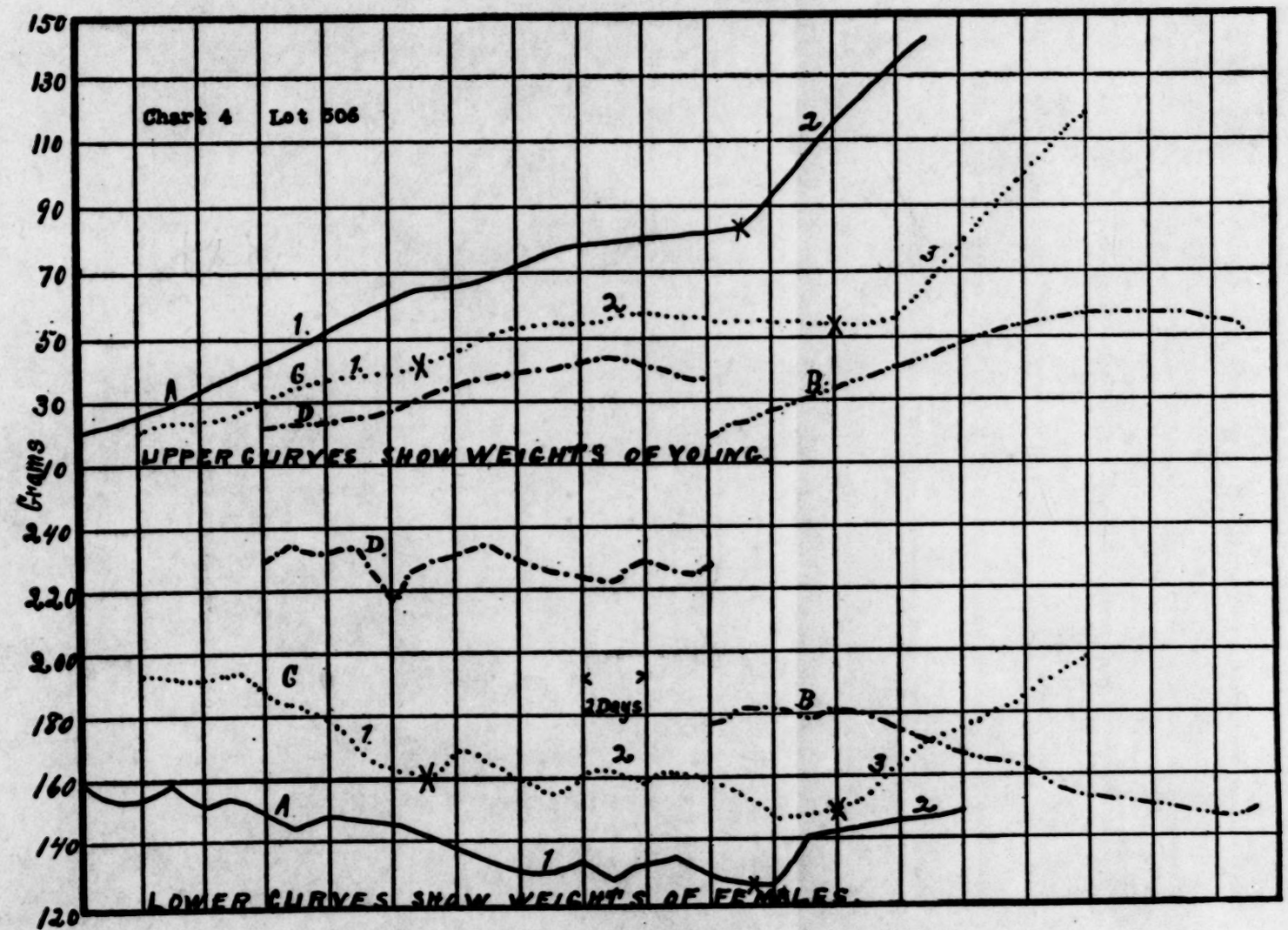

CHART 4.

(E. V. McCollum, N. Simmonds, and W. Pits: Unidentified Dietary Factors.)

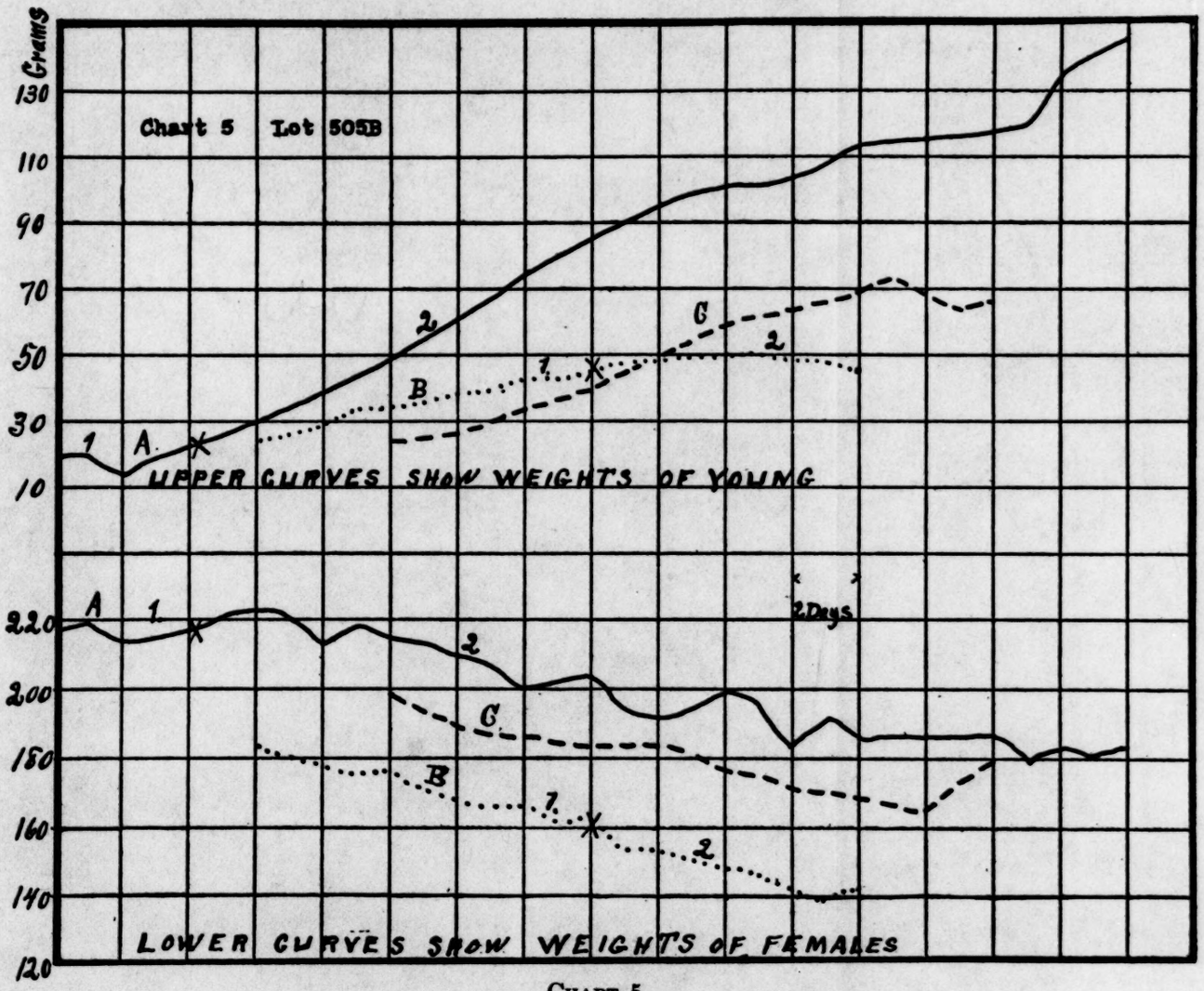

CHART 5.

(E. V. McCollum, N. Simmonds, and W. Pits: Unidentified Dietary Factors.)

In Chart 5, Lot 505 B, Period 1, the ration of all three rats was the same, and lacked the fat-soluble A and derived the water-soluble B from the cold water extract of 50 gm. of rolled oats for each 100 gm. of ration. In Period 2 the changes made in the rations of Rats A and B were as follows.

Rat A was changed to the alcoholic extract of 10 gm. of wheat embryo per 100 gm. of ration as a source of the dietary B, and butter fat was added to supply the A. Her young did not grow at the maximum rate, but their growth was continuous and they were successfully weaned. We are inclined to the belief that 18 per cent of casein is hardly sufficient for the lactating rat and to attribute the somewhat slow growth to this cause.

In Period 2, Rat B was given butter fat and the cold water extract of rolled oats was increased so that each 100 gm. of ration carried the extract of 150 gm. of oats. There was surprisingly little benefit from these changes. Cold water extract of rolled oats may not furnish the B, or as is more probable, the other extractives (salts, etc.) from so large an amount of oats may have had an unfavorable effect on the mother. We did not investigate this matter further.

The ration employed (Lot 505 B) had the following composition:

Casein	18.0
Dextrin	76.3
Salt mixture (185)	3.7
Agar-agar	2.0

The dextrin carried the cold water extract of 50 gm. of rolled oats.

For the composition of Salt Mixture 185, see above.

Period 2, Rat A. 5 per cent of butter fat was introduced in place of an equivalent amount of dextrin.

Rat B received the same ration as did Rat A, Period 2, except that the water-soluble B was furnished by the cold water extract of 150 gm. of rolled oats. This extract was carried by the dextrin.

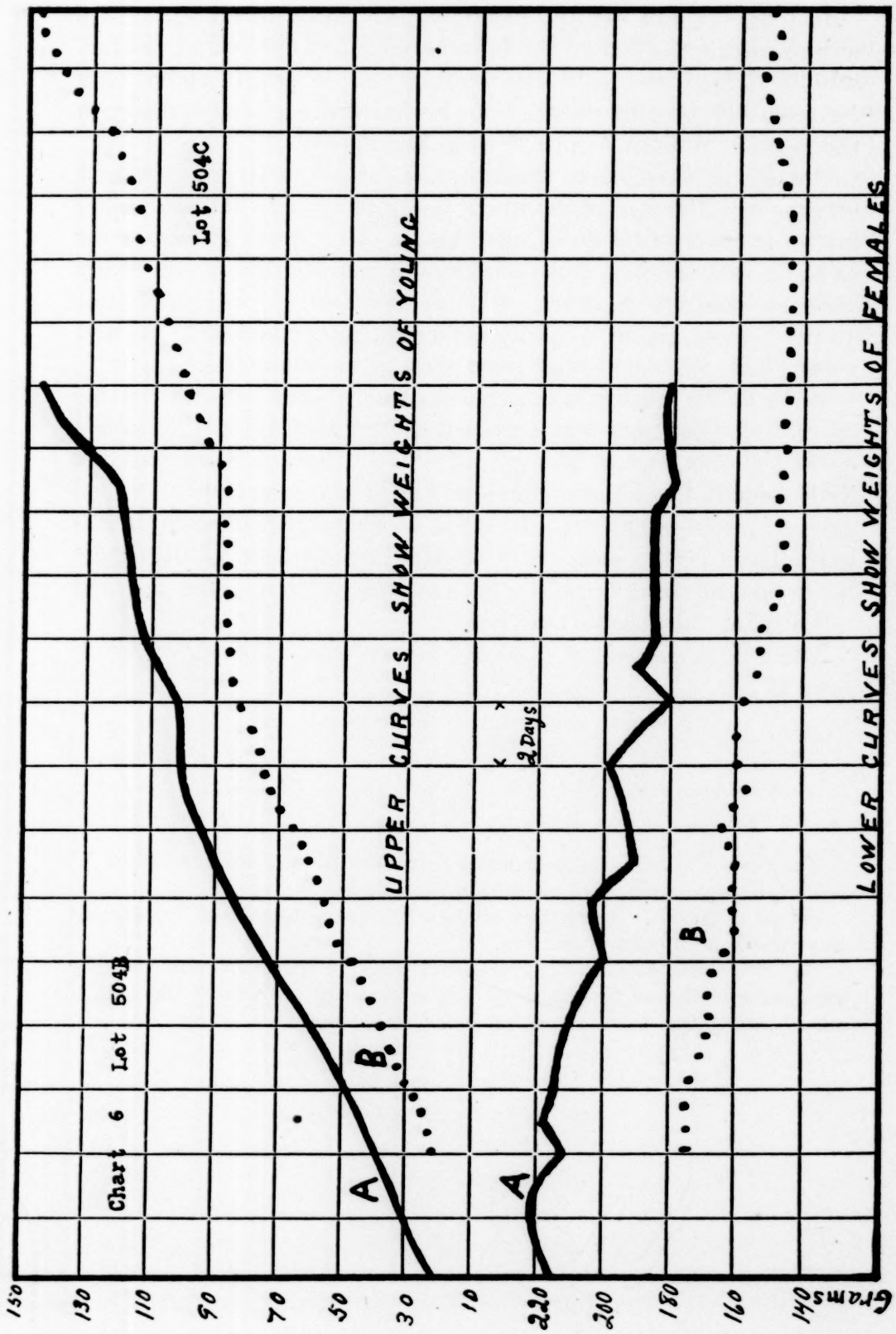
Chart 6 Lot 504B
Lot 504C
UPPER CURVES SHOW WEIGHTS OF YOUNG
LOWER CURVES SHOW WEIGHTS OF FEMALES
A
B
A
B
2 Days
Grams
150
130
110
90
70
50
30
10
220
200
180
160
140

Chart 6.

Chart 6, Lot 504 B, Rat A, shows fairly satisfactory growth in the young and practically maintenance of body weight of the mother on our basal ration to which both the fat-soluble A and water-soluble B were added at the beginning of the nursing period. The failure of the young in these two litters as well as those in Chart 5, Rat A, to grow at the maximum rate is probably to be attributed to the inadequacy of 18 per cent of casein as a source of protein for the lactating rat.

Rat B, Lot 504 C, whose ration was similar to that of Rat A except that double the amount of alcoholic extract of wheat embryo was contained in it, failed to promote the growth of her young any better because of the high content of the dietary factor B, indicating that the extract of 10 gm. of embryo which was contained in other rations of this series, supplied an adequate amount of this substance.

The ration (Lot 504 B) consisted of:

Casein	18.0
Dextrin	71.3
Salt mixture (185)	3.7
Butter fat	5.0
Agar-agar	2.0

The dextrin carried the alcoholic extract of 10 gm. of wheat embryo.

For the composition of Salt Mixture 185, see above.

For Lot 504 C the ration was the same as that of 504 B except that the dextrin carried the alcoholic extract of 20 gm. of wheat embryo.

THE INFLUENCE OF ETHER ANESTHESIA ON AMINO-ACIDS OF BLOOD SERUM.

By ELLISON L. ROSS.

(From the Department of Pharmacology, Northwestern University Medical School, Chicago.)

(Received for publication, August 7, 1916.)

The pharmacology of ether used as an anesthetic is the subject of many conflicting theories and statements. The numerous phases of the subject still need minute and extensive investigation before any clear conception of the action of this much used compound can be secured. We have already made studies of the dextrose and diastase content of the blood as affected by ether anesthesia.[1] In this case the amino-acid content of the blood serum has been the subject of investigation.

EXPERIMENTAL WORK.

Five groups of dogs were used in this work. Each group was treated differently. Group I was fed ground lean beef daily for 1 week. Each dog received 250 gm. about noon each day. The operative work was done on the dogs the day after the last feeding. A sample of blood was taken from the jugular vein just before anesthesia. The ether was given by forcing air saturated with ether into a nose mask for the dog. After surgical anesthesia had continued for 15 minutes another sample of blood was taken. The amino-acid content of the blood was determined by the method of Van Slyke[2] with the modification that the sample and reagents were increased five times and the determinations made on 2 cc. volume of the total filtrate which had been made up to 10 cc. volume. Several determinations were made on each sample and the average was taken as the correct result. The results of the work on Group I are given in Table I.

[1] Ross, E. L., and McGuigan, H., *J. Biol. Chem.*, 1915, xxii, 497.

[2] Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 385.

TABLE I.

Influence of Ether after a Week of Meat Feeding.

Dog.	Mg. of amino N per cc. of serum.		
	Before ether.	After 15 min. of anesthesia.	Change.
1	0.066	0.064	−0.002
2	0.046	0.049	+0.003
3	0.069	0.063	−0.006
4	0.111	0.110	−0.001
5	0.111	0.101	−0.010
Average.....	0.0806	0.0774	−0.0032 −4.0 per cent

Group II was made up of five dogs which were fed on a low protein diet for 1 week previous to the test. They were fed 200 gm. of a mixture of crackers 2 parts and lard 1 part. The animals otherwise were treated in the same way as those of Group I. The results are given in Table II.

TABLE II.

Influence of Ether after a Week of Low Protein Diet.

Dog.	Mg. of amino N per cc. of serum.		
	Before ether.	After 15 min. of anesthesia.	Change.
1	0.070	0.074	+0.004
2	0.022	0.024	+0.002
3	0.037	0.034	−0.003
4	0.063	0.063	−0.000
5	0.049	0.046	−0.003
Average.....	0.0482	0.0482	−0.000 0.0 per cent

Group III consisted of five dogs which had not been receiving a special diet before the tests. Half an hour before bleeding the first time each dog was given a mixture containing 100 gm. of sugar, 16 gm. of starch, and water. After bleeding they were anesthetized and treated as were the dogs of the preceding groups. The results are given in Table III.

TABLE III.

Influence of Ether ½ Hour after Carbohydrate Feeding.

Dog.	Mg. of animo N per cc. of serum.		
	Before anesthesia.	After 15 min. of anesthesia.	Due to ether.
1	0.072	0.069	−0.003
2	0.054	0.055	+0.001
3	0.069	0.061	−0.008
4	0.062	0.067	+0.005
5	0.068	0.070	+0.002
Average.....	0.065	0.0644	−0.0006 −0.9 per cent

Group IV consisted of ten dogs which had not been previously dieted. They were bled, fed 200 gm. of ground lean beef each, bled at the end of half an hour after feeding, anesthetized, and after 15 minutes of surgical anesthesia, bled again. Amino-acid determinations were made on the samples. The results are given in Table IV.

TABLE IV.

Influence of Ether ½ Hour after Meat Feeding.

Dog.	Mg. of amino N per cc. of serum.			Changes.	
	Before feeding.	½ hr. after feeding.	After anesthesia.	Due to feeding.	Due to ether.
1	0.061	0.061	0.051	0.000	−0.010
2	0.123	0.112	0.116	−0.011	+0.004
3	0.071	0.072	0.057	+0.001	−0.015
4	0.118	0.130	0.124	+0.012	−0.006
5	0.046	0.051	0.041	+0.005	−0.010
6	0.149	0.127	0.138	−0.022	+0.011
7	0.049	0.046	0.038	−0.003	−0.008
8	0.121	0.118	0.130	−0.003	+0.012
9	0.071	0.076	0.062	+0.005	−0.014
10	0.144	0.140	0.140	−0.004	0.000
Average..	0.0953	0.0933	0.0897	−0.0020 2.1 per cent	−0.006 3.9 per cent

Group V was made up of undieted dogs. They were bled, fed a pound of ground lean beef each, bled after 4 hours, anesthetized, and bled again after 15 minutes of anesthesia. The results are given in Table V.

TABLE V.

Influence of Food and Ether.

Dog.	Mg. of amino N per cc. of serum.			Changes.	
	Before feeding.	4 hrs. after feeding.	After 15 min. anesthesia.	Due to feeding.	Due to ether.
1	0.116	0.168	0.160	+0.052	−0.008
2	0.105	0.159	0.148	+0.054	−0.011
3	0.091	0.136	0.144	+0.045	+0.008
4	0.081	0.179	0.135	+0.098	−0.044
5	0.097	0.141	0.124	+0.044	−0.017
Average..	0.098	0.1566	0.1422	+0.0586	−0.0144
				+59.8 per cent	−9.2 per cent
				+47.8 per cent*	−4.6 per cent*

*Averages with the omission of Dog 4, in which there was an exceptionally large increase due to feeding, and a correspondingly large decrease due to ether.

DISCUSSION.

The results given in Table I show the influence of ether anesthesia on the amino-acid content of the blood serum of dogs which had been dieted for 1 week on lean beef. There was a small drop in four out of five cases. The average change in the group was a drop of 4.0 per cent of the original content of the serum.

The change in the amino-acid content of the blood serum due to anesthesia of dogs fed on practically no protein for 1 week is given in Table II. In one case there was no change, in two there was an increase, and in two a decrease. The average of all five changes was zero.

The figures given in Table III show the change, due to ether, in the amino-acid content of blood serum of dogs which half an

hour previous to anesthesia received considerable amounts of carbohydrates. The results are variable. Three out of five show an increase due to ether but the average of all shows a decrease of 0.9 per cent. Compared with the wide individual variations, the average change is negligible.

Table IV gives the results of feeding meat half an hour before anesthesia. The change in the amino-acid content of the blood serum due to the taking of food and to the administration of ether is determined. The individual changes due to both food and ether are variable; some are positive and some are negative. The average change due to meat feeding was a fall of 2 per cent and that due to ether anesthesia following a fall of 3.9 per cent.

Table V contains data on the amino-acid change of the blood serum due first to 4 hours of meat digestion and second to ether anesthesia following. The average change for all five dogs due to meat digestion was 59.8 per cent increase and that due to ether anesthesia was a fall of 9.2 per cent. Dog 4 made an enormous increase due to feeding and a very exceptionally large decrease due to the ether. Averaging the results of the other four dogs, there was an increase due to meat digestion of 47.8 per cent and a decrease due to anesthesia of 4.6 per cent. This probably is more near the changes that would be found in the average normal dog. Van Slyke and Meyer[3] obtained similar results.

It is interesting to note that very roughly the amount of decrease of amino-acids due to ether anesthesia follows the initial amino-acid content of the blood. This is shown in the following table compiled from the preceding tables:

Table.	Amino N before ether.	Fall.
		per cent
II	0.0482	0.0
III	0.0650	0.9
IV	0.0953	3.9
I	0.0806	4.0
V	0.0980	4.6–9.2

[3] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 409.

CONCLUSIONS.

The amino-acid content of the blood serum of dogs is affected as follows. 1. Not materially changed in half an hour by feeding either carbohydrates or meat. 2. Increased considerably in 4 hours after feeding a pound of meat to each dog. 3. Not materially decreased by 15 minutes of ether anesthesia, first, after a week of meat diet, second, after a week of low protein diet, third, half an hour after feeding meat, or fourth, half an hour after feeding carbohydrates and fat. The decrease at most is not above 4.0 per cent. 4. Decreased 4.6 to 9.2 per cent after an amino-acid rise due to 4 hours of meat digestion.

STUDIES ON GROWTH.

III. THE COMPARATIVE VALUE OF LARD AND BUTTER FAT IN GROWTH.

By CASIMIR FUNK and ARCHIBALD BRUCE MACALLUM.*

(From the General Memorial Hospital, Harriman Research Laboratory, Roosevelt Hospital, New York, and the Department of Pathological Chemistry, University of Toronto.)

(Received for publication, August 3, 1916.)

In our first communication[1] of this series, we advanced the opinion that we were dealing with a problem very similar to, if not identical with, beri-beri. Our main objective was to ascertain the simplest dietary conditions necessary to enable a young rat to reach maturity. Subsequently we found[2] that artificial diets containing butter, without yeast or similar vitamine-containing substances, are insufficient to promote growth in young rats; and at that time the question whether butter could be replaced by lard with the same ultimate success was left open. We have carried out experiments of longer duration, employing both lard and butter as the fat fraction of these diets, and submit results which enable us to form a more definite opinion as to the relative value of these two fats.

Our experience demonstrates that there are wide variations depending on the constitution of the individual rats. Every rat taken indiscriminately is not suitable for this class of work. As a matter of fact in experiments carried out in Toronto 80 per cent of the rats purchased from dealers were rejected on account of physical defects not apparent before the initiation of the experiment. A second complication is a diminished resistance to infection, which follows the use of all artificial diets. The meager knowledge we possess of the pathological conditions

* Senior Fellow in Medical Research.

[1] Funk, C., and Macallum, A. B., Jr., *Z. physiol. Chem.*, 1914, xcii, 13.

[2] Funk and Macallum, *J. Biol. Chem.*, 1915, xxiii, 413.

in rats may lead to a condemnation of the diet; whereas actually the condition could be remedied without change of diet, if we were able to recognize its nature. As an example of this, rats on artificial diets frequently contract an eye infection which can be treated with a certain degree of success by an application of a few drops of zinc sulfate solution. If untreated this condition is accompanied by loss in weight, becomes acute, and terminates fatally.

The first series of experiments were carried out on diets containing lard as the fat component, and dried powdered yeast. Rats on this diet grew normally for 60 to 90 days, but eventually displayed symptoms (bleeding from the eyes, nose, and ears, petechiæ and hemorrhages under the skin of the tail) which might be regarded as scorbutic. This terminated fatally if no change of diet was effected. When moist yeast was substituted for the dried preparation the rats could be kept for 150 days and attained approximately adult size. Autolyzed yeast was equally efficient in this respect. Similar results were obtained on addition of orange juice to the drinking water, although orange juice itself has neither growth-promoting nor maintaining properties, unless supplemented by yeast.

Diets in which butter partially or wholly replaces lard have a slight superiority over those containing lard, which is more than can be explained by the antiscorbutic properties of the butter. Rats on yeast and butter diets often show the eye affection regarded by most of the investigators as characteristic of dietary deficiencies, and we are convinced that none of the artificial diets so far investigated can be compared with a normal dietary in its efficiency for growth. This deficiency introduces an additional complication and must be taken into account in subsequent investigations.

EXPERIMENTAL.

The methods of preparing the diets were very much the same as those described in our earlier publications. The experiments varied slightly as to their conditions in New York and Toronto but the ultimate results were identical. The charts and tables are representative of the different groups of experiments.

Experiment I.

Rats 49, 50, 51, and 52 (Fig. 1) were kept on diets containing dried yeast and lard for about 68 days. At that time a deficiency was noticed which, in previous experiments, led to the death of all the rats and could not be corrected by a larger supply of dried yeast. Then fresh moist pressed yeast was substituted, the deficiency disappeared, and the rats attained approximately adult weight.

A second series, Rats 61 and 62 (Fig. 2), were placed on diets containing lard for 44 days, being changed to a diet of butter and dried yeast after this period. No increment in growth was noticed as the result of this change.

In the butter experiments Rats 53 and 54 (Fig. 1) have also shown a marked improvement on changing the yeast from the dried to the moist form, more especially as regards their external appearance. Rats 47 and 48 (Fig. 2) were kept on diets with butter and dried yeast and these have also developed symptoms which persisted when the diets were substituted by lard and autolyzed yeast. Rats 59 and 60 (Fig. 2), exceptionally healthy specimens, were kept on butter-containing diet for 44 days and then changed[3] to diet with lard and autolyzed yeast, for a longer period than was indicated in the chart, without the rate of growth being modified. On several occasions rats showing deficiency on a lard-containing diet were placed on a butter diet with the hope of relieving the symptoms. The improvement which resulted from this change was only temporary and several rats died after being kept 30 days on butter.

In all the experiments a marked improvement resulted when a diet of a different composition was given or even from a fresh preparation of the same diet. This might indicate that the diets lose part of their nutritive value when stored for lengthy periods.

[3] Encircled numbers on Fig. 2 indicate the point at which that diet was begun.

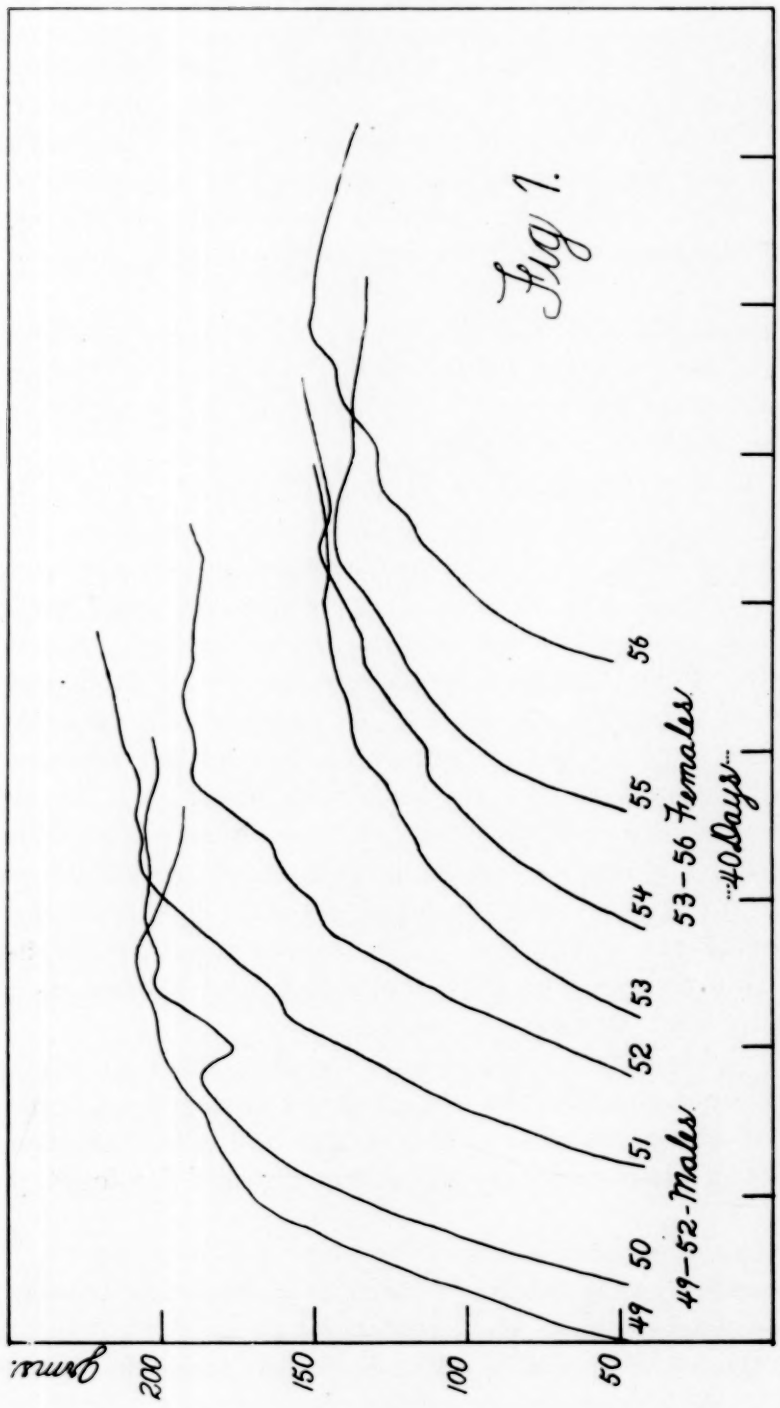

FIG. 1. Rats 49, 50, 51, and 52 were kept on diets containing lard and dried yeast. The animals recovered when moist yeast was substituted for dried yeast. Rats 53 and 54 were kept on butter and dried yeast. Here also a marked recovery was noticed on changing to the wet form of yeast. Rats 55 and 56 were kept on a diet containing casein purified according to the method of McCollum; no advantage of the use of this method is noticeable.

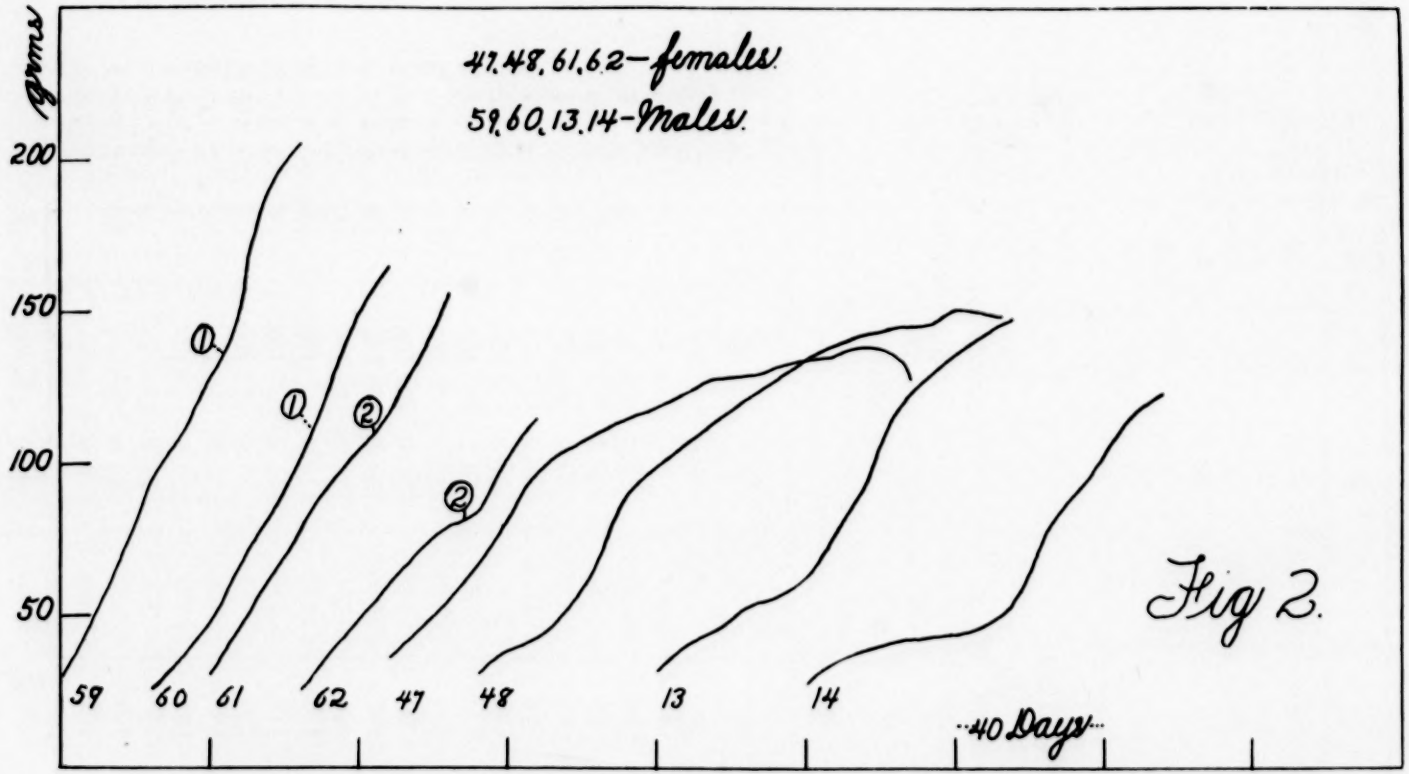

FIG. 2. Rats 59 and 60 have been kept on a diet of butter and dried yeast, which diet was then changed at the point indicated in the curve to lard and autolyzed yeast without any effect on the rate of growth. Rats 61 and 62 were kept on lard and then changed to butter without any effect on growth. Rats 47 and 48 were kept on butter and dried yeast and developed signs of food deficiency which persisted when the diet was changed to lard and autolyzed yeast. Rats 13 and 14 were kept on a diet containing casein which had been autoclaved. The rats failed to grow on this diet but recovered when 1 cc. of orange juice was added, which indicates that this deficiency was not due to chemical changes in the casein.

Diets (Gm.).

	1.	2.	3.	4.	5.
Casein	22	22	22	22	22
Sugar	10	10	10	10	10
Starch	30	27	27	29	27
Lard	30	30	30	30	30
Salt	3	3	6	6	6
Agar	2	2	2	2	2
Yeast (dry)	3	6	3		
Yeast (moist) equal to				1 of dry yeast.	3 of dry yeast.

Rats 49 and 50. Males.
0– 68 days Diet 2
69– 98 " " 4
99–150 " " 5

Rats 51 and 52. Males.
0– 52 days Diet 1
53– 68 " " 3
69– 98 " " 4
99–150 " " 5

Days.	Weight.		Average food intake per day.	Weight.		Average food intake per day.
	49.	50.		51.	52.	
	gm.	*gm.*	*calories*	*gm.*	*gm.*	*calories*
0	44	46		41	46	
4	55	58	77.4	62	55	78.2
8	81	85	92.4	72	65	81.3
12	96	100	103.2	91	82	67.2
16	104	110	105.7	102	91	97.1
20	119	124	110.3	110	100	102.2
24	132	137	110.5	119	110	106.8
28	146	150	110.0	132	120	100.3
32	150	158	111.0	138	128	100.8
36	162	163	109.3	148	138	112.2
40	170	174	114.6	159	147	116.2
44	173	176	114.3	160	150	115.2
48	177	180	111.1	163	150	117.5
52	178	184	95.4	170	158	108.6
56	182	188	110.8	176	161	120.3
60	185	191	103.8	178	163	106.6
64	186	176	78.7	175	164	96.9
68	184	182	90.0	192	167	120.7
76	199	200	115.9			116.3
84	201	201	98.5	206	190	108.5
100	207	205	85.0	209	192	91.0
120	200	205	105.7	210	190	103.7
140	193	201	76.3	216	186	85.4
150	192	203	75.8	221	190	67.4

Diets (Gm.).

	1.	2.	3.	4.
Casein	22	22	22	22
Sugar	10	10	10	10
Starch	30	27	29	27
Butter fat	18	18	18	18
Lard	12	12	12	12
Salt	3	3	6	6
Agar	2	2	2	2
Yeast (dry)	3	6		
Yeast (moist) equal to			1 of dry yeast.	3 of dry yeast.

Rats 53 and 54. Females.

0–52 days Diet 1
53–68 " " 2
69–100 " " 3
101–150 " " 4

Days.	Weight.		Average daily food intake.
	53.	54.	
	gm.	*gm.*	*calories*
0	43	41	
4	54	55	74.3
8	64	63	66.2
12	78	75	72.0
16	80	80	77.0
20	85	87	73.5
24	90	92	76.8
28	96	95	81.1
32	100	101	79.2
36	106	106	88.9
40	112	112	87.3
48	112	112	87.2
56	119	116	83.7
64	123	126	95.2
84	138	135	79.8
100	144	146	76.5
120	149	147	79.2
150	147	153	92.8

Diets (Gm.)

	1.	2.	3.
Casein	22	22	22
Sugar	10	10	10
Starch	28	30	23
Butter	30	30	
Lard			30
Agar	2	2	2
Salts	2	2	2
$NaHCO_3$	1	1	1
Yeast (dry)		3	
Yeast (autolyzed) equal to	1.5 of dry yeast.		3 of dry yeast.

Rats 47 and 48. Females.
0– 16 days Diet 1.
16–123 " " 2.
123–140 " " 3.

Days.	Weight.		Average daily food intake.
	47.	48.	
	gm.	*gm.*	*gm.*
0	36.5	31	
4	41	34	6.2
8	45.5	39	9.5
12	51.5	43	9.1
16	54.5	42.5	9.1
20	58	45	10.0
24	67.5	50.5	11.2
28	76	56.5	12.1
32	85	66	14.1
36	91	78	13.6
40	99	91	14.2
44	102	93	12.5
48	105	98	13.5
52	108	100.5	14.5
56	111	107	14.3
60	114.5	113.5	15.0
64	115.5	115	16.9
68	119	122	15.2
72	119	120	14.0
76	121.5	125.5	15.3
80	124.5	129.5	15.3
84	127	134.5	17.1
88	128	134.5	14.6
92	126.5	135	14.1
96	128.5	139	16.1
100	130.5	141	14.7
108	134	144.5	16.6
116	133	145	16.9
124	135	146.5	16.6
132	139.5	154	14.6
140	128	149.5	14.0

Diets (Gm.).

	1.	2.
Casein	22	22
Starch	23	30
Sugar	10	10
Butter		30
Lard	30	
Agar	2	2
Salts	2	2
$NaHCO_3$	1	1
Yeast (dry)		3
Yeast (autolyzed) equal to	3 of dry yeast.	

Rats 59 and 60. Males.
0–44 days Diet 2.
45–64 " " 1.

Rats 61 and 62. Females.
0–44 days Diet 1.
45–64 " " 2.

Days.	Weight.		Average daily food intake.	Days.	Weight.		Average daily food intake.
	59.	60.			61.	62.	
	gm.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*
0	30.5	25	6.7	0	30.5	25.5	6.6
4	40	31	8.2	4	38	32	9.3
8	47.5	35.5	9.7	8	46.5	38	9.3
12	59	42.5	11.2	12	55.5	43	10.7
16	70	48	13.8	16	62.5	48	12.5
20	81.5	58.5	14.8	20	71.5	55	12.6
24	94	68	16.2	24	80.5	61.5	14.6
28	106	75.5	17.2	28	87.5	67	14.2
32	114	82.5	20.1	32	93.5	71	16.1
36	127.5	90	20.8	36	99.5	76	14.9
40	139.5	100.5	23.8	40	105	80.5	14.2
44	155	113	24.9	44	111.5	84.5	14.2
48	167	124	23.7	48	115	83	16.6
52	180	138	21.8	52	123	96	17.6
56	192	150	22.0	56	132	100	15.2
60	199	157	21.1	60	142	104.5	17.1
64	205	165	21.1	64	156.5	115	

Experiment II.

The casein preparation used in this series was purified by washing, following the method of McCollum and Davis.[4] In

[4] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 231.

this paper the authors claim that purification of casein by boiling with alcohol destroys some of the amino-acids and results in loss of its nutritive properties. The results in this case (Rats 55 and 56, Fig. 1) were identical with those which were obtained with casein purified by extraction with hot alcohol. This latter method was used in purifying the casein in the first experiment.

Diets (Gm.).

	1.	2.	2.
Casein (McCollum)	22	22	22
Sugar	10	10	10
Starch	27	29	27
Lard	30	30	30
Salt	3	6	6
Agar	2	2	2
Yeast (dry)	6		
Yeast (moist) equal to		1 of dry yeast.	3 of dry yeast.

Rats 55 and 56. Females.
0– 59 days Diet 1.
60– 87 " " 2.
88–140 " " 3.

Days.	Weight.		Average daily food intake.
	55.	56.	
	gm.	*gm.*	*calories*
0	48	52	
4	67	71	75.1
8	80	82	92.2
12	89	91	88.4
16	96	96	77.2
20	104	102	86.0
24	107	104	74.9
28	109	109	94.3
32	115	116	79.8
40	120	120	88.4
60	140	133	86.3
80	144	143	78.4
100	136	150	79.9
140	133	136	61.9

Another series, of which Rats 13 and 14 (Fig. 2) are representatives, received casein which had been autoclaved for 1 hour at 15 pounds' pressure, according to McCollum and Davis. On this diet the rats failed to grow, but after 28 days 1 cc. of fresh orange juice was added, and normal growth was resumed. It seems probable that the impaired value of heated casein is not due so much to the destruction of amino-acids as to the loss of its anti-scorbutic properties.

Diets (Gm.)

	1.	2.
Casein	22	22
Sugar	10	10
Starch	30	29
Lard	30	30
Salts	3	3
Agar	2	2
Yeast (dry)	3	4

Rats 13 and 14. Males.
0– 14 days Diet 1.
15– 96 " " 2.
28–100 " 1 cc. orange juice.

Days.	Weight.		Average daily food intake.
	13.	14.	
	gm.	*gm.*	*gm.*
0	33.3	27.2	
4	40.1	33.7	6.2
8	41.4	37.1	6.0
12	44.6	39.8	7.1
16	46.4	39.4	7.9
20	50.6	41.4	7.2
24	54.3	42.0	8.1
28	54.6	41.5	6.5
32	55.1	42.0	4.7
36	63.1	47.0	8.2
40	62.1	44.0	6.8
44	70.8	46.2	8.4
48	77.1	46.2	10.6
52	87.1	51.8	12.2

Rats 13 and 14. Males—*Continued.*

Days.	Weight.		Average daily food intake.
	13.	14.	
	gm.	*gm.*	*gm.*
56	93.0	54.6	12.4
60	102.0	64.2	12.9
64	119.3	78.6	15.6
68	126.0	89.3	15.1
72	130.0	91.6	15.4
76	130.9	98.6	14.9
80	137.4	103.8	15.1
84	140.0	112.4	14.8
88	144.7	117.0	11.2
92	145.0	119.8	14.2
96	147.0	123.0	11.4

SUMMARY.

The failure of rats to grow on a lard and yeast diet is partially due to the development of scorbutic symptoms. These can be relieved to a marked degree by using moist instead of dried yeast and still more so by using moist yeast and butter. Even in the latter case the existing deficiencies are not entirely corrected, since many rats decline on this diet. Rats which fail on lard do not always recover on a diet containing butter. It seems also possible that yeast on account of its high content in purines, and perhaps other constituents, is not an ideal addition in experiments of long duration, even in spite of its marked growth-promoting power. The impaired nutritive value of heated casein does not seem to be due to destruction of amino-acids but to destruction of vitamines.

STUDIES ON GROWTH.

IV. THE ACTION OF YEAST FRACTIONS ON THE GROWTH OF RATS.

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The close relationship existing between the beri-beri and growth problems suggests the possibility of a fractionation of the active substance along lines already used in the investigation of beri-beri. Accordingly phosphotungstic acid was selected for the first attempt to separate out a physiologically active fraction which would stimulate the growth of young rats. The experimental difficulties which have repeatedly been emphasized in the investigation of the beri-beri vitamine, due to instability of this substance, were also encountered in the study on growth. The physiological activity of the different fractions diminishes with each manipulation and both the problems of beri-beri and of growth will not be solved until more suitable methods for the isolation of vitamine are available.

The results obtained so far clearly indicate that the growth-promoting substance is analogous to and possibly identical with the beri-beri vitamine and can be almost entirely precipitated with phosphotungstic acid. Subsequent fractionation of the residue obtained from the decomposed precipitate with silver nitrate and also with silver nitrate and baryta has shown that the precipitate containing purine bases and the filtrate from the silver nitrate and baryta precipitation are entirely negative as to their growth-promoting action; whereas the substances pre-

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cipitated with silver nitrate and baryta possess traces of the activity of the initial phosphotungstic acid precipitate. The experimental evidence indicates that considerably larger quantities of vitamines are necessary for stimulating growth than for curing beri-beri, and the losses occurring during fractionation are more apparent in the former than in the latter case. However, it must be admitted that while it is uncertain whether these two substances are chemically different, the results obtained do not exclude such a possibility. Lloyd's reagent, as recommended by Seidell,[1] has also been used as a precipitant without much success, as the rats on the filtrate have also shown increments in growth.

In the first instance autolyzed yeast was slightly acidified with hydrochloric acid and completely precipitated with phosphotungstic acid, carefully avoiding an excess of this reagent. After allowing the mixture to stand for 24 hours, the precipitate was collected on a Buchner funnel and repeatedly washed with a cold solution of phosphotungstic acid containing hydrochloric acid. The precipitate was then decomposed by the method described by Van Slyke,[2] with a mixture of amyl alcohol and ether and hydrochloric acid, only a small quantity of the precipitate remaining unchanged. After filtration of this small fraction the aqueous extract was evaporated *in vacuo* and the residue made up to a known volume and mixed in the diet in quantities calculated from the amount of autolyzed yeast necessary to promote growth. However, the quantity of this fraction had to be doubled and even tripled in order to obtain satisfactory results. The phosphotungstic acid filtrate was worked out in a similar way. This process offers the advantage that the yeast fraction is completely freed from substances soluble in lipoid solvents. The purine fraction was obtained from the phosphotungstic acid precipitate fraction by precipitation with silver nitrate and subsequent decomposition with sulfuretted hydrogen. The filtrate from the purine bases was precipitated with baryta in the usual way and the precipitate decomposed, freed from traces of baryta, evaporated, and the residue mixed with

[1] Seidell, A., *U. S. Public Health Report*, No. 325, 1916.
[2] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

the diet. The filtrate from the fraction containing vitamines was reprecipitated with phosphotungstic acid and the precipitate obtained after thorough washing, decomposed with amyl alcohol and ether. The results with the purine fraction and also with the silver nitrate-baryta filtrate are not included in this paper as they were entirely negative. The effect of the silver nitrate-baryta fraction was not sufficiently marked to encourage further investigation. The diet contained lard as the fat constituent, and 1 per cent sodium bicarbonate was added to neutralize the hydrochloric acid present in this fraction. Orange juice to the extent of 1 cc. a day was added to the drinking water to prevent the onset of scorbutic symptoms.

A large number of rats were kept on the above diets, especially on the phosphotungstic precipitate and filtrate and the records of only a few were selected for publication, in order to save space. Rats 9 and 10 were kept on phosphotungstic acid filtrate throughout the experiment. Rats 11 and 12 were changed after 34 days to the diet containing the phosphotungstic precipitate fraction which was followed by an improvement warranting the view that the growth-promoting substance is contained in this precipitate (Fig. 2 b).

Diets (Gm.).

	1.	2.
Casein	22	22
Sugar	10	10
Starch	23	23
Lard	30	30
Agar	2	2
Salts	2	2
$NaHCO_3$	1	1
Phosphotungstate precipitate		10
" filtrate	10	

Rat 9 (male) and Rat 10 (female).
0–96 days Diet 1.
28–100 " 1 cc. orange juice.

Rats 11 and 12. Males.
0–34 days Diet 1.
35–100 " " 2.
76–100 triple vitamine addition.

Days.	Weight.		Average daily food intake.	Days.	Weight.		Average daily food intake.
	9.	10.			11.	12.	
	gm.	gm.	gm.		gm.	gm.	gm.
0	35.2	41.8		0	37.9	34.2	
4	42.8	47.3	9.5	4	46.2	41.8	7.6
8	43.0	47.3	7.8	8	45.8	44.2	5.5
12	44.6	49.3	7.0	12	42.8	45.3	7.6
16	46.8	55.4	8.7	16	45.3	45.8	7.8
20	46.2	56.6	7.5	20	46.9	45.7	7.7
24	46.6	54.7	7.8	24	46.2	46.5	6.6
28	46.5	52.8	5.5	28	44.3	45.0	5.4
32	42.9	49.9	7.4	32	40.4	39.4	4.7
36	37.8	41.6	7.8	36	39.8	37.2	5.6
40	36.8	41.6	7.7	40	50.5	48.2	9.1
44		41.8	6.6	44	52.2	50.0	8.9
48		43.9	5.3	48	54.8	57.0	8.0
52		44.0	5.3	52	58.0	60.7	8.7
56		43.8	3.8	56	63.5	67.3	11.0
60		41.6	3.5	60	66.3	71.0	12.4
64		44.6	3.5	64	66.1	70.3	8.8
68		41.0	3.2	68	70.6	75.7	11.1
72		43.5	3.5	72	75.0	82.6	9.9
76		42.9	3.4	76	76.0	84.8	11.9
80		45.0	3.0	80	77.6	91.1	11.1
84		42.2	3.1	84	75.0	85.2	9.6
88		44.1	2.7	88	79.4	90.0	8.8
92		43.0	3.2	92	78.5	90.9	4.4
96		41.0	2.9	96	74.7	91.7	6.4
100				100	70.0	76.3	6.4

A second series of experiments was carried out on pressed yeast which had been heated with 10 per cent sulfuric acid at 90–95° for 6 hours. The hydrolysate was filtered, diluted with an equal volume of water, and precipitated with phosphotungstic acid. After standing for 24 hours the precipitate was filtered at the pump and well washed with 5 per cent sulfuric acid.

The precipitate was decomposed in the ordinary way with baryta. The final solution, slightly acid, was neutralized with sodium carbonate, carefully avoiding an excess, distilled *in vacuo*, standardized, and definite quantities were added to the diet. The

filtrate of the phosphotungstic acid precipitation was treated in a similar way.

The diet containing the substances precipitated by phosphotungstic acid was fed to four rats (Rats 80 to 83, Fig. 1) and enabled them to double their original weight after 32 to 36 days. This is about double the time required when yeast is the source of vitamines and the depreciation is due to the fractionation with the precipitating reagent.

Two rats (84 and 85, Fig. 2 a) were fed the diet containing the residue from the phosphotungstic filtrate. After 28 days they had added only a third to their original weight and had all the external symptoms of an acute deficiency. Then the diet with the precipitate was substituted and in 11 days they rapidly doubled their original weight and presented a normal appearance.

Diets (Gm.).

	1.	2.
Casein	22	22
Sugar	10	10
Starch	24	24
Fat (lard)	30	30
Salt	6	6
Agar	2	2
Residue phosphotungstic acid precipitate equal to	6	Dried yeast.
Residue phosphotungstic acid filtrate equal to		6

Rats 80, 81, and 82. Males.
Rat 83. Female.
0–36 days Diet 1.

Days.	Weight.		Average daily food intake.	Weight.		Average daily food intake.
	80.	81.		82.	83.	
	gm.	*gm.*	*calories*	*gm.*	*gm.*	*calories*
0	24	25		20	30	
4	31	33	29.8	23.5	37	27.8
8	36	40.5	33.5	28	43	32.8
12	36	43	35.7	29	45	33.3
16	38	44.5	40.5	29	48	40.5
20	40	48	28.6	31	51	38.3
24	42	49	30.1	34	54	30.2
28	44.5	52	27.8	38	57	35.0
32	46	54	28.7	40	59	27.4
36	49	56	25.0	42.5	61	29.8

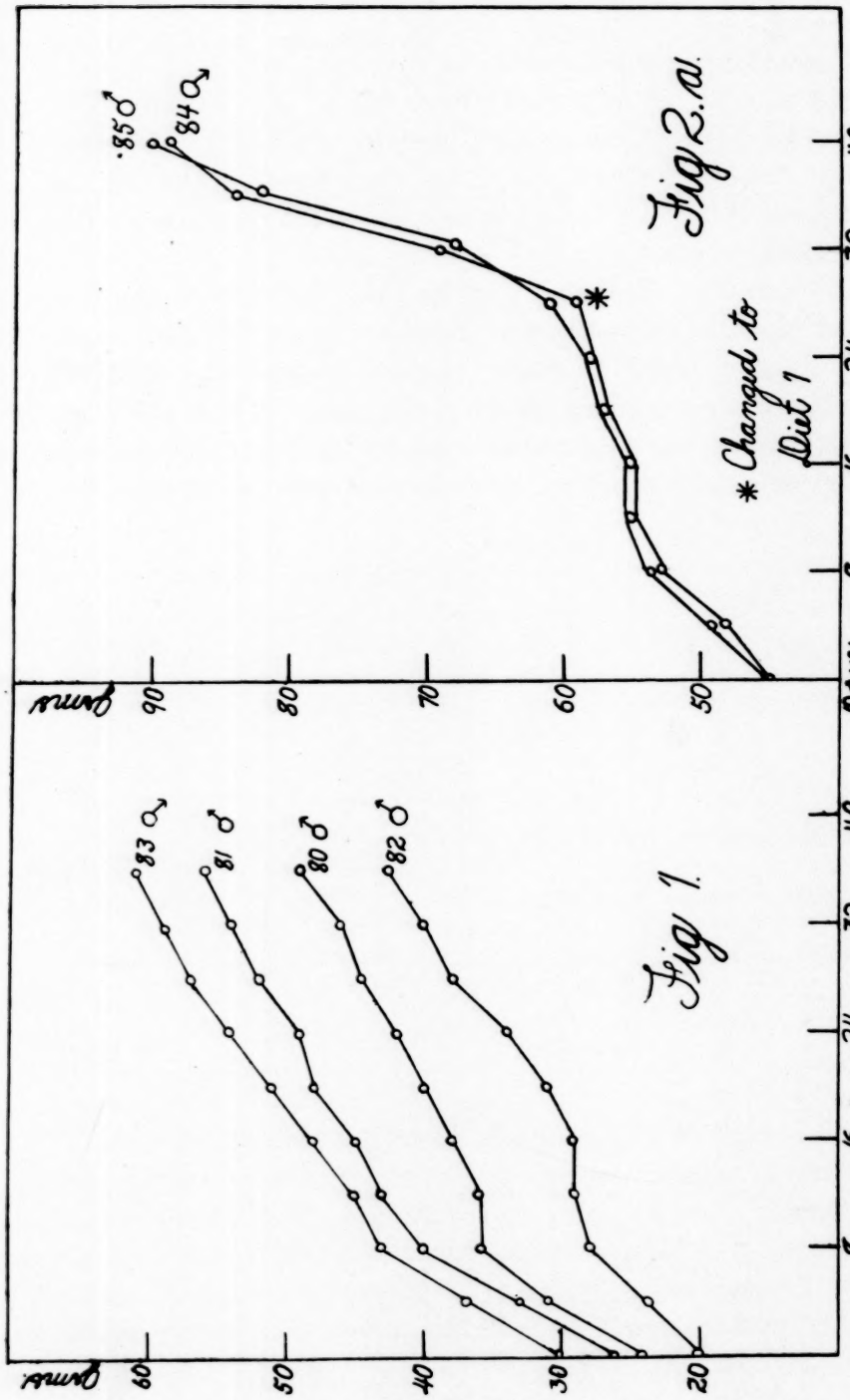

FIG. 1. Rats 80, 81, 82, and 83 were kept on a diet containing decomposed phosphotungstic acid precipitate from yeast.

FIG. 2 a. Rats 84 and 85 were kept first on a diet containing the residue from phosphotungstic filtrate of yeast. The marked deficiency was corrected when this addition was changed on the point marked on the chart to the corresponding precipitate.

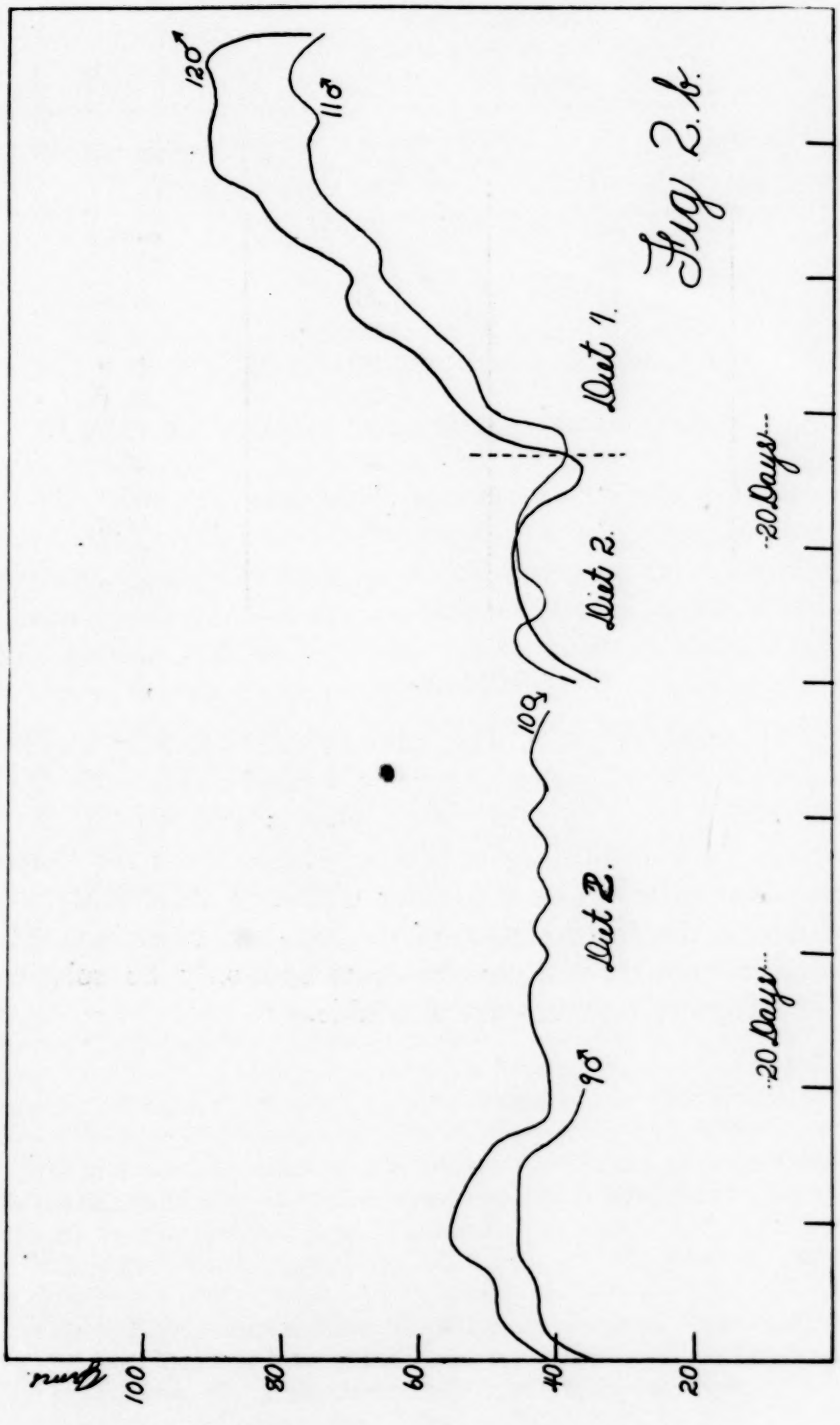

FIG. 2b. Rats 9 and 10 were kept on a diet containing phosphotungstic acid filtrate throughout the experiment. Rats 11 and 12 were changed to phosphotungstic precipitate at the point marked on the chart, with a marked improvement in growth and general appearance.

Rat 84. Female.
Rat 85. Male.
0–28 days Diet 2.
29–39 " " 1.

Days.	Weight.		Average daily food intake.
	84.	85.	
	gm.	*gm.*	*calories.*
0	45	45	
4	48	49	28.6
8	53	54.5	46.3
12	55	55	42.9
16	55	55	35.9
20	57	57	50.9
24	58	58	48.6
28	59	61	47.9
32	69	68	54.6
36	84	82	70.3
39	89	90	66.3

SUMMARY.

The fractionation of yeast with phosphotungstic acid shows that the growth-promoting substance is carried down with the precipitate and a large part of its activity is lost during the fractionation. The instability of this substance when fractionated with silver salts presents greater difficulty than that experienced during the fractionation of the beri-beri vitamine. It seems possible that both these problems can only be solved when more adequate methods are available.

THE REACTION BETWEEN AMINO-ACIDS AND CARBOHYDRATES AS A PROBABLE CAUSE OF HUMIN FORMATION.*

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The study of the black substances obtained when proteins are hydrolyzed in strong acid solution is of great interest at the present time on account of their bearing on the natural melanins and on the quantitative determination of certain amino-acids in proteins. Grindley[1] and his coworkers state that humin nitrogen causes an error in the analysis for amino-acids of common foodstuffs when the Van Slyke amino nitrogen determination is directly applied to them. This view on theoretical grounds was also expressed by Hart and Bentley.[2] It is therefore very important to know more about the structure and mode of formation of these compounds.

Mulder[3] was the first to show that albumins separate flocculi of a brown or black color on being boiled with concentrated hydrochloric or sulfuric acids. Hausmann[4] made similar observations with globin. Samuelly[5] pointed out that the formation of these "artificial melanins" or "melanoidins" might be a secondary reaction between amino-acids and carbohydrates. Maillard[6] conducted experiments along this line and found a

* The work described in this article forms part of a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Wisconsin.

[1] Grindley, H. S., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 2762.

[2] Hart, E. B., and Bentley, W. H., *J. Biol. Chem.*, 1915, xxii, 477.

[3] Mulder, G. J., in Mann, G., Chemistry of the Proteids, London, 1906, 87.

[4] Hausmann, W., *Z. physiol. Chem.*, 1900, xxix, 140.

[5] Samuelly, F., *Beitr. chem. Phys. u. Path.*, 1902, ii, 355.

[6] Maillard, L. C., *Compt. rend. Acad.*, 1912, cliv, 66.

number of them reacted with sugars. His experiments, however, were carried on in aqueous solutions at a very high concentration and temperature and it is doubtful whether under these conditions the reaction is similar to what takes place in the formation of either the "natural" or artificial melanins. It will be shown later in this paper that not all the amino-acids found reactive by Maillard reacted at all at low concentration in water. Gortner and Blish[7] made the important discovery that when tryptophane is boiled with sugar in 22.9 per cent hydrochloric acid solution 86 per cent of its nitrogen is converted into humin nitrogen. They conclude from their experiments that tryptophane alone is responsible for humin formation. Grindley and his coworkers[1] disagree with this conclusion since they found evidence that other amino-acids give the same reaction.

In view of these conflicting statements and in the hope that the study of the reaction between amino-acids and carbohydrates would throw some light on the structure and mode of formation of the humin substances, it was thought worth while to determine: (1) Which amino-acids react with carbohydrates under a given set of conditions. (2) Whether different sugars behave alike toward the same reactive amino-acid. (3) What group of the reactive amino-acids takes part in the reaction.

REVIEW OF THE LITERATURE.

Udránszky[8] and Hoppe-Seyler[9] have shown that when sugar is boiled with acids humin substances are formed, and if boiled in the presence of a nitrogenous material, the humins may also contain nitrogen. Udránszky found, for example, that glucose and urea boiled together in strong hydrochloric acid solution formed humins which contained about 6.73 per cent nitrogen.

Samuelly[5] was the first to study the behavior of humins, or "melanoidins," towards oxidizing and reducing agents. He prepared his "melanoidins" from commercial serum albumin according to Schmiedeberg's[10] method modified by himself. He subjected his product to the action of

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[7] Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630. After this work was completed and sent for publication another article by Gortner on humin formation appeared (*J. Biol. Chem.*, 1916, xxvi, 177). In this article Gortner admits that amino-acids other than tryptophane may be involved in humin formation, which is in harmony with the results reported here.

[8] Udránszky, L. v., *Z. physiol. Chem.*, 1888, xii, 33.

[9] Hoppe-Seyler, F., *Z. physiol. Chem.*, 1889, xiii, 66.

[10] Schmiedeberg, O., *Arch. exp. Path. u. Pharm.*, 1897, xxxix, 1.

HI and PI_3 in a closed tube kept for 8 hours at 200–210°C. Among other products he obtained pyrrol, as detected by the color reaction with pine shavings, and either pyridine or piperidine or some derivative of either one of these bases. Ammonia was also evolved in large amounts. None of the reduction products obtained as above gave indol or skatol on fusion with alkalies, while the humins before reduction gave on similar treatment an unmistakable odor of both. Samuelly also tried reduction with zinc dust in a current of hydrogen. From this treatment he obtained pyridine, pyrrol-like substances, skatol, and small amounts of an aromatic compound of the benzaldehyde series. The same investigator prepared humin substances from some amino-acids and glucose. He[11] heated for 18 hours a mixture of 10 gm. of glucose, 50 cc. water, 15 cc. concentrated HCl (sp. gr. 1.19), and sufficient amount of the different amino-acids so as to have in the solution 0.7 gm. of nitrogen. He tried ammonium chloride, urea, acetamide, glycocoll, aspartic acid, cystine, and tyrosine. In each case he found nitrogen in the melanin. No attempt was made, however, to determine whether the humin nitrogen formation was due to adsorption or to a definite reaction. It is interesting to note that all of the humins so prepared gave off pyrrol on dry distillation with zinc dust, no pyridine, and on fusion with alkalies only the humin prepared from tyrosine produced an odor of indol. Nencki[12] and Berdez[13] obtained similar results with the natural melanins. By alkali fusion these authors obtained from tumor melanin, indol, skatol, volatile fatty acids, hydrocyanic acid, succinic acid, and other unidentified products. Pyrrol was obtained on dry distillation, and after heating the melanin to 300°C. for some time, upon addition of an alkali pyridine was detected.

Gortner and Blish[7] heated pure zein, plus tryptophane, plus carbohydrate in 22.86 per cent HCl, and obtained 16.5 per cent of the total nitrogen of the mixture in the humin form. When tryptophane alone was heated with sugar in acid solution 86.56 per cent of its nitrogen was found in the humins. On the other hand, when histidine plus zein was heated with acid only 0.51 per cent of the total nitrogen was found in the humins. This amount was almost the same as that obtained when zein alone was heated in acid. They did not try, however, heating zein, plus histidine, plus sugar, in acid. Among the conclusions these authors draw from their experiments are the following: (1) The humin nitrogen belongs to "no amino-acids other than tryptophane." (2) "The reaction involved . . . . is probably the condensation of an aldehyde with the —NH group of the tryptophane nucleus." (3) Histidine can be eliminated "as a factor in the formation of humin nitrogen."

Grindley and Slater[1] have tried to apply the Van Slyke amino nitrogen determination directly to the analysis of feedingstuffs. As is to be expected,

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[11] Samuelly, *Beitr. chem. Phys. u. Path.*, 1902, ii, 383.

[12] Nencki, M., and Sieber, N., *Arch. exp. Path. u. Pharm.*, 1887, xxiv, 17.

[13] Berdez, J., and Nencki, M., *Arch. exp. Path. u. Pharm.*, 1886, xx, 346.

on account of the high carbohydrate content of these, the humin fraction in their nitrogen distribution is very high, varying from 3.85 per cent in blood meal to 15.79 per cent in alfalfa hay, expressed as *per cent* of the total nitrogen. In discussing the origin of these humin substances these investigators disagree with the conclusion arrived at by Gortner and Blish, that the humin nitrogen of protein hydrolysis has its origin exclusively in the tryptophane nucleus, since they have obtained "results that clearly indicate that in addition to tryptophane a number of other amino-acids when gently boiled with 20 per cent HCl for 24 to 30 hours in the presence of pure glucose give humin nitrogen. Preliminary experiments show that under the above treatment 4.7 to 6.3 per cent of the total nitrogen of lysine and cystine respectively is separated as humin nitrogen."

Since Bourquelot and Bertrand discovered tyrosinase, this enzyme has received much attention from a great number of investigators. Only that part of the work relating to the action of tyrosinase on the different amino-acids and related substances will be reviewed here.[14]

The effect of tyrosinase on tyrosine is described by Bertrand.[15] A solution of tyrosine to which an extract of tyrosinase is added first becomes red, then inky black, and finally deposits a black precipitate. He proved definitely that atmospheric oxygen is essential to the change by conducting experiments *in vacuo* and in the air. Von Fürth and Schneider[16] used the blood (hemolymph) of the pupæ of a butterfly, *Deiciphila elpenor.* They separated the enzyme from the other substances in the blood by fractional precipitation with ammonium sulfate. It was found to give a yellowish red substance with pyrochatechol; with hydroquinone it gave a red solution, which then became turbid and finally deposited a considerable brownish precipitate. It also acted on adrenalin, giving a dirty brown color. Oxyphenylethylamine became yellowish brown and finally gave an olive-colored precipitate. But tyrosinase has no action on casein itself. The same authors isolated the black substance produced from tyrosine by the tyrosinase of *Deiciphila* pupæ and determined its elementary composition. Below is given a comparison between the percentage composition of this black substance and of tyrosine respectively:

	Black substance (humin) from tyrosine. *per cent*	Tyrosine. *per cent*
C	55.66	59.60
H	4.45	6.08
N	13.74	7.74
O	26.37	26.58

[14] Bourquelot and Bertrand, G., *Bull. Soc. Mycol.*, 1896, xii, 18. A complete list of references up to the time of its publication is found in Kastle's Oxidases, *Bull. Hyg. Lab.*, 59.

[15] Bertrand, G., *Compt. rend. Soc. biol.*, 1896, cxxii, 1215.

[16] Von Fürth, O., and Schneider, H., *Beitr. chem. Phys. u. Path.*, 1901, i, 229.

In the formation of this "artificial melanin" from tyrosine there is an increase in the nitrogen content from 7.74 to 13.74. Such an increase is only conceivable in one of two ways; either there is a breaking up of the tyrosine molecule, or some other nitrogenous substance besides tyrosine takes part in the formation of the melanin. The latter must be the case since tyrosinase, being but a weak oxidizing agent, would be unable to break down the benzene nucleus. The nitrogenous compound that took part in the reaction must evidently have come from the tyrosinase preparation itself. This black product also yields a skatol-like odor on fusion with alkali. In connection with the wide distribution of tyrosinase in both the vegetable and animal kingdom the following is quoted from Kastle's monograph:

"Von Fürth and Schneider are therefore of the opinion that probably wherever melanotic pigments occur in the living tissues of the lower and higher animals they originate as the result of the action of appropriate enzymes on substances of aromatic nature. They point out in this connection that Salkowski and Jacoby have shown independently that tyrosine results from the autolysis of various animal tissues. It would seem likely, therefore, that in the formation of melanotic pigments two ferments are jointly concerned: one, an autolytic ferment capable of splitting off tyrosine or a similar aromatic complex from the protein molecule, and the other tyrosinase, which transforms the tyrosine into melanin."

But one of the most interesting phases of the investigations on tyrosinase is that relating to its effect on the products of protein degradation and related substances. Bertrand and Rosenblatt[17] have found that this enzyme acts equally well upon racemic and *l*-tyrosine. Chodat and Staub[18] discovered that albumoses do not give a red color with tyrosinase but glycyltyrosine anhydride gives such a coloration. In a later article[17] these authors observed that glycine, leucine, and alanine retard the action of tyrosinase; that dipeptides such as tyrosine anhydride, and glycyltyrosine anhydride produce yellow substances which do not become black as does tyrosine itself. When, however, glycine, leucine, or alanine is present, a red coloration similar to that resulting from tyrosine is obtained: glycyltyrosine anhydride with glycine gives a rose color changing to bluish green; with alanine the color is deeper red, with leucine deep brown. But their most striking discovery is that phenylalanine is not acted on by tyrosinase. This, however, acts readily on *p*-cresol, less readily on *m*-cresol, and still less readily on *o*-cresol. In fact these same authors observed that the enzyme acts most readily on the para-homologues of phenol. Amino-acids like glycine increase the rapidity of the action of tyrosinase on *p*-cresol, producing a violet color which ultimately becomes blue. Bertrand undertook to investigate the action of tyrosinase

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[17] Bertrand, G., and Rosenblatt, M., *Compt. rend. Soc. biol.*, 1908, cxlvi, 304.

[18] Chodat, R., and Staub, *Arch. Sc. Phys. Nat.*, 1907, xxiii, 265; xxiv, 172.

from wheat bran on compounds analogous to tyrosine and to phenylalanine; that is, compounds with and without the phenolic hydroxyl group. Thus he found phenylalanine, phenylethylamine, phenylmethylamine, phenylaminoacetic acid, phenylpropionic acid, phenylacetic acid, alanine, and glycocoll produced no coloration at all. On the other hand the following compounds with phenolic hydroxyl groups produced coloration as follows:

Tyrosine	Grenadine-red, inky black.
p-Hydroxyphenylethylamine	Grenadine-red, olive-black.
p-Hydroxyphenylmethylamine	Orange-yellow, orange-red, clear maroon.
p-Hydroxyphenylamine	Orange, mahogany-red, brown.
p-Hydroxyphenylpropionic acid	Orange-yellow, grenadine-red, brown.
p-Hydroxybenzoic acid	Rose, orange, yellow.
p-Cresol	Yellow, orange, red.
Phenol	Yellow. orange, red, brown.

He concludes, therefore, that tyrosinase acts only on those compounds containing a phenolic group.

In 1907 Abderhalden and Guggenheim[19] published an interesting article on the effect of tyrosinase from *Russula delica* on tyrosine, tyrosine-containing polypeptides and other related substances. They observed that glycocoll, *d*-alanine, *d*-valine, *l*-proline, *d*-serine, *d*-*l*-isoserine, and *l*-phenylalanine retard the action of tyrosinase on tyrosine only slightly unless present in very large concentrations. The largest concentration used was molar; *l*-aspartic acid and *d*-glutamic acid, however, even when present at a concentration of 0.01 molar retard the action considerably. The same authors found that the enzyme has no effect on diiodotyrosine, *l*-phenylalanine, *l*-proline, or cystine. But *l*- and *d*-tyrosine, homogentisic acid, and tryptophane showed a color change. Particularly interesting was the case of *d*-tryptophane. The authors state that at first they thought that the coloration with tryptophane may be due to traces of tyrosine. They, however, used a very pure product. They repeated their experiment but always came to the same result. Furthermore, they tried the effect of tyrosinase on solutions of tryptophane-containing polypeptides and found development of color. They therefore conclude that this coloration must not be ascribed to the presence of traces of tyrosine. Still more interesting is the fact that neither indol nor skatol were found to produce coloration. Abderhalden and Guggenheim in the same article describe the effect of tyrosinase on polypeptides containing tyrosine. The color developed in these cases is modified to some extent by the nature of the amino-acid combined with the tyrosine in the polypeptide. Addition of some amino-acids were also found either to accelerate or to retard the action of tyrosinase on the polypeptide. Thus proline acceler-

[19] Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.*, 1907–08, liv, 331.

ates considerably the action of the oxidase on glycyl-*l*-tyrosine anhydride, while aspartic acid and glutaminic acid retard the action. On the other hand halogen derivatives of the polypeptides were not acted upon by tyrosinase. The same authors also found, as did Bertrand, that tyrosinase acts on phenol, giving a brown color, which was modified by amino-acids. Thus glycocoll plus phenol gave a cochineal color, while proline and phenol gave violet. The authors finally concluded that the amino-acids, when present, apparently take part in the production of the pigment. In a later article[20] these same authors point out that tyrosinase acts on adrenalin with the rapid production of a red color and ultimately dark red flocculi. All three isomers of adrenalin are affected with equal rapidity.

It is to be regretted that in none of the above cited contributions was either arginine, histidine, or lysine tried. It is hoped that this omission will be filled in the near future.

EXPERIMENTAL.

The fact that zein, when boiled with glucose in 22.68 per cent hydrochloric acid solution, increases its humin nitrogen from 0.56 to 1.84 per cent indicates, as Grindley and his coworkers[1] suggested, that other amino-acids besides tryptophane take part in nitrogenous humin formation. Only a small per cent of some of these amino-acids may take part in this formation so that only by working with the individual amino-acids is it possible to determine whether the humin nitrogen was due to a definite reaction or to an adsorption. Again it is only by working under approximately the same set of conditions that it is possible to detect differences in behavior between the different amino-acids. The following procedure was, therefore, adhered to as consistently as practicable.

The amino-acid, plus sugar, plus 50 cc. of water or hydrochloric acid solution of the specified strength was heated for 48 hours in a 300 cc. Kjeldahl flask on a sand bath. The flask was provided with a reflux condenser made from a large test-tube fitted with cork and tubings for a current of cold water. After heating, the digestion mixture was neutralized with the calculated amount of sodium hydroxide solution. The salt thus formed coagulated most of the precipitate that may have existed in a colloidal state in the solution. The mixture was then filtered into 200 cc. graduated flasks and the humin was washed repeatedly

[20] Abderhalden and Guggenheim, *Z. physiol. Chem.*, 1908, lvii, 329.

with boiling water until the flask was filled to the mark. This amount of washing was found to be sufficient to remove almost all of the adsorbed amino-acid which could be removed by this treatment alone. The humin with the filter paper was then Kjeldahled. The filtrate was either Kjeldahled or Van-Slyked or used for both determinations. 25 cc. portions were taken for the Kjeldahl and 10 cc. portions for the Van Slyke determination.

The nitrogen content of the amino-acids was determined either by Kjeldahl's or by Van Slyke's method or by both. The *per cent* of nitrogen was the only thing used to establish the identity and purity of the compounds.

The following amino-acids were furnished by Professor Hart:

Amino-acid.	Nitrogen. Found. *per cent*	Theoretical. *per cent*
Alanine	15.70	15.75
Cystine	11.2–11.6	11.67
Tyrosine	7.67	7.72
Lysine hydrobromide ($2C_6H_{14}N_4O_2 . HBr . H_2O$)	14.72	14.32
Tryptophane	6.44 (Amino N)	6.86
Phenylalanine*	6.95	6.94

* The phenylalanine was kindly furnished by Dr. T. B. Osborne of New Haven.

The following amino-acids were prepared:

Amino-acid.	Nitrogen. Found. *per cent*	Theoretical. *per cent*
Leucine, from zein	10.90	10.70
Proline, from zein, also from gelatin	12.20	12.17
	(No amino N)	
Glutaminic acid, from gliadin	7.17	7.64
Arginine (free) from gelatin	29.95	32.20
Amino N	7.70	8.04
Histidine dihydrochloride, from blood	17.25	18.42
Amino N	5.76	6.14

In the preparation of the above amino-acids the directions given in Abderhalden's *Arbeitsmethoden* were followed. The *per cents* of nitrogen found for arginine and histidine respectively were not quite up to the theoretical, but since the amino nitrogen was almost one-fourth of the total in the arginine sample and one-third in the histidine, it was evident that the samples of both

these amino-acids were free from other amino-acids, their low total nitrogen content being due to moisture. The nitrogen determinations of these amino-acids were made on the same day that the experiments on humin formation were started.

Due to the scarcity of material it was found impossible to recrystallize some of the amino-acids in order to obtain as pure a product as could be desired.

The results are shown in the following table. All the experiments were in duplicate and average figures are given.

The results show that neither alanine nor leucine give humin nitrogen. Glutaminic acid when boiled with sugar in 2 per cent acid solution yields some humin nitrogen, but none in 20 per cent acid. Attention is called to the fact that glutaminic acid on heating even at the concentration used seems to form pyrrolidon carboxylic acid readily, as evidenced by the loss of activity of its amino nitrogen in Experiments 13, 14, and 16. Such a formation does not take place in strong acid. Phenylalanine yields about 1.65 per cent of its nitrogen in the humin in 20 per cent acid solution. Proline does not give humin nitrogen with glucose with 20 per cent acid, but seems to react to some extent with xylose and fructose in 4.15 per cent acid solution. Cystine with 20 per cent HCl yields about 3.1 per cent humin nitrogen and the noteworthy fact about this amino-acid is the deeply colored filtrate it produced. The same was observed with the filtrate from the tyrosine-sugar experiments. As much as 15 per cent of tyrosine nitrogen may be converted into humin nitrogen.

The cases of the three hexone bases are particularly interesting. When boiled with sugars in 20 per cent HCl solution all three yield some of their nitrogen as humin nitrogen. Arginine and lysine, with sugar, give more deeply colored filtrates than histidine. If the deep coloration of the filtrate indicates reaction, then it must be stated definitely that in the case of cystine, tyrosine, arginine, and lysine in 20 per cent HCl, the humin nitrogen is due to a reaction and not to an adsorption. Another fact that supports the contention that a definite reaction is responsible for humin formation at least in the case of tyrosine is that phenylalanine gives but little humin nitrogen. If this were a case of adsorption, then there should probably be no differ-

TABLE I.

Record of Results.

Experiment No.	Treatment.	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
	Alanine.								
1	0.089 gm. + 20 per cent HCl.....	0.0		13.88	13.98	0.10		13.98	Filtrate colorless.
2	0.089 gm. + 0.720 glucose + 20 per cent HCl.....	0.0		13.81	13.98	0.17		13.98	" light yellow.
3	0.089 gm. + 0.600 gm. xylose + 20 per cent HCl*								
4	0.089 gm. + 0.720 gm. fructose + 20 per cent HCl.....	0.0		14.00	13.98	−0.02		13.98	" " "
5	0.089 gm. + 2.0 gm. glucose + 20 per cent HCl.....	0.0		14.01	13.98	−0.03		13.98	" " "
6	0.0415 gm. + 0.720 gm. glucose + 20 per cent HCl.....	0.0		6.51	6.55	0.04		6.55	" " "
	Leucine.								
7	0.131 gm. + 4.15 per cent HCl.....	0.0		14.36	14.36	0.0		14.36	" colorless.
8	0.131 gm. + 0.720 gm. glucose + 4.15 per cent HCl.....	0.0		14.33	14.36	0.03		14.36	" light yellow.
9	0.131 gm. + 0.720 gm. fructose + 4.15 per cent HCl.....	0.0		14.30	14.36	0.06		14.36	" " "

* Determination lost.

10	0.131 gm. + 0.720 gm. glucose + water	0.0		14.40	14.36	−0.04	14.36	Filtrate light yellow.	
11	0.131 gm. + 0.720 gm. glucose + 20 per cent HCl	0.0		14.40	14.36	−0.04	14.36	"	" "
	Glutaminic acid.								
12	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	0.10 0.35 per cent		28.60	28.60	0.00	28.70	"	" "
13	0.400 gm. + 2 gm. glucose + 20 per cent HCl	0.46 1.65 per cent		22.00	28.24	6.70	28.70	"	" "
14	0.162 gm. + 0.720 gm. glucose + water	0.05 0.03 per cent		4.47	11.55	7.13	11.60	"	colorless.
15	0.162 gm. + 0.720 gm. glucose + 20 per cent HCl	0.00		11.80	11.60	−0.20	11.60	"	light yellow.
16	0.162 gm. + water	0.00		4.58	11.60	7.02	11.60	"	colorless.
	Phenylalanine.								
17	0.4 gm. + 2.0 gm. glucose + 20 per cent HCl	0.46 1.65 per cent		27.50	27.32	−0.18	27.80	"	light yellow.
18	0.4 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00		28.00	27.80	−0.20	27.80	"	colorless.
19	0.2015 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00		28.00	27.80	−0.20	27.80	"	"

TABLE I—*Continued.*

Experiment No.	Treatment.	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
	Proline.								
20	0.115 gm. + 4.15 per cent HCl.....	0.00	14.07				14.07 100.1 per cent	14.00	Filtrate almost colorless.
21	0.115 gm. + 0.720 gm. glucose + 4.15 per cent HCl.....	0.00	14.12				14.12 100.9 per cent	14.00	" light yellow.
22	0.115 gm. + 0.600 gm. xylose + 4.15 per cent HCl.....	0.53 3.66 per cent	13.10				13.63 97.5 per cent	14.00	" " "
23	0.115 gm. + 0.720 gm. fructose + 4.15 per cent HCl.....	0.33 2.36 per cent	13.98				14.31 102.0 per cent	14.00	" " "
24	0.380 gm. + 2.0 gm. glucose + 20 per cent HCl.....	0.00						–	" " "

25	0.6315 gm. + 2.0 gm. glucose + 20 per cent HCl	0.00							Filtrate light yellow.
	Cystine.								
26	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.25 2.9 per cent							Filtrate yellowish brown.
27	0.400 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00							Filtrate almost colorless.
28	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.36 3.1 per cent	43.20	43.60	43.44	−0.16	44.56 99.4 per cent	44.8	Filtrate yellowish brown.
29	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl	1.36 3.1 per cent	43.12	43.60	43.44	−0.16	44.48 99.3 per cent	44.8	Filtrate yellowish brown.
30	0.200 gm. + 2.0 gm. glucose + 0.1 cent HCl	0.00	20.84				20.84 89.5 per cent	23.3	Filtrate light yellow.
31	0.200 gm. + 2.0 gm. proline + 2.0 gm. glucose + 20 per cent HCl	1.61*	45.70				47.31 99.4 per cent	47.70	Filtrate yellowish brown.

*3.38 per cent of total N or 6.9 per cent of cystine N.

TABLE I—*Continued.*

Experiment No.	Treatment.	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino-N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
	Tyrosine.								
32	0.1586 gm. + 20 per cent HCl	0.00		12.22	12.22			12.22	Filtrate brownish.
33	0.1586 gm. + 0.720 gm. glucose + 2.5 per cent HCl	0.20		12.01	12.02	0.01		12.22	" yellowish brown.
34	0.1586 gm. + 0.720 gm. glucose + 20 per cent HCl	0.84		11.40	11.38	−0.02		12.22	" brownish.
35	0.1586 gm. + 2.0 gm. glucose + 20 per cent HCl	1.33		10.62	10.89	0.27		12.22	" "
36	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	4.64		26.00	26.06	0.06		30.70	" "
	Arginine.								
37	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	2.34 1.97 per cent	115.00	28.20	28.90	0.70	117.34 98.4 per cent	119.5	" yellowish brown.
38	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl	2.77 2.33 per cent	115.50	28.70	28.95	0.25	118.27 99.0 per cent	119.5	" "
39	0.400 gm. + 2.0 gm. glucose + water	0.35 0.29 per cent	115.00	21.70	28.90	7.20	115.35 96.2 per cent	119.5	" brownish carmine.

	Lysine.								
40	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl.....	1.54 2.62 per cent	57.5	57.50	57.50	0.00	58.94 100.5 per cent	58.90	Filtrate yellowish brown.
41	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl.....	1.47 2.50 per cent	57.4	57.00	57.40	0.40	58.77 99.9 per cent	58.90	" " "
42	0.2 gm. + 1.0 gm. glucose + water.	0.28 0.48 per cent	28.3	23.70	28.60	4.90	28.58 98.0 per cent	29.20	" carmine.
	Histidine.								
43	0.1915 gm. + 20 per cent HCl.....			14.00	14.00			42.00	" light yellow.
44	0.1915 gm. + 0.360 gm. glucose + 4.15 per cent HCl.....	0.61 1.45 per cent		12.19	13.80	1.61		42.00	" " "
45	0.1915 gm. + 0.720 gm. glucose + 20 per cent HCl.....	0.77 1.84 per cent		12.81	13.74	0.93		42.00	" " "
46	0.1915 gm. + 0.600 gm. xylose + 4.15 per cent HCl.....	1.08 2.58 per cent		12.38	13.62	1.24		42.00	" " "

TABLE I—*Concluded.*

Experiment No.	Treatment.	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
47	0.1915 gm. + 0.600 gm. xylose + water	0.68 1.62 per cent		11.53	13.80	2.27		42.0	Filtrate yellowish brown.
48	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	0.80 1.16 per cent	68.00	23.20	22.70	−0.50	68.80 99.8 per cent	69.0	" light yellow.
49	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl	0.77 1.12 per cent	67.20	23.40	22.40	−1.00	67.90 98.4 per cent	69.0	" " "
50	0.200 gm. + 1.0 gm. glucose + water	0.00	35.40	11.68	11.80	0.12	35.40 100.0 per cent	35.4	" " "
51	0.200 gm. + 1.0 gm. fructose + water	0.21 0.32 per cent	33.30	8.70	11.10	2.30	33.51 94.6 per cent	35.4	" deep brown.
	Tryptophane.								
52	0.1514 gm. + 1.0 gm. glucose + 20 per cent HCl	13.82 71.0 per cent	5.44	2.73	2.72	−0.01	19.26 98.8 per cent	19.50	" almost colorless.

ence in behavior between tyrosine and phenylalanine. In aqueous or very weak acid solution arginine, histidine, and lysine evidently react with sugar as indicated by the highly colored solutions produced and by the loss of activity of a large fraction of their amino nitrogen. Thus, when arginine plus glucose was boiled in water there was a very deep coloration of the solution (Experiment 39), and at least 25 per cent of the amino nitrogen became inactive towards nitrous acid. Lysine behaved similarly (Experiment 42), 17 per cent of the amino nitrogen becoming inactive towards nitrous acid. Histidine acted likewise (Experiments 47 and 51), 16.2 per cent of its amino nitrogen becoming inactive. These facts show that in the cases of histidine and arginine the α-amino nitrogen takes part in the reaction. In the case of lysine it is difficult to establish which amino group is reactive, since at the time the amino nitrogen in the filtrate was determined the temperature in the laboratory was about 35°C. and at this temperature it was found that both the α- and the ϵ-amino group of lysine react with nitrous acid in 5 minutes, as may be seen in the amino nitrogen determination of the filtrate (Experiments 40 to 42). It is to be noted that in these cases some loss of nitrogen also took place. It may be that during the reaction some ammonia was given off.

The result with tryptophane is in agreement with the work of Gortner and Blish[7] in that a greater portion of the tryptophane nitrogen is converted into humin. The strength of the acid used here and the different procedure followed may account for the difference in the *per cent* of tryptophane nitrogen found in the humin which according to the above named authors was 86 per cent while in these experiments only about 71 per cent was obtained. Due to a lack of material it was impossible to repeat the experiment with tryptophane.

In order to determine which atomic groupings in tyrosine, cystine, and tryptophane were responsible for humin formation, the humin from each one of these amino-acids was dissolved in 0.1 N alkali and Van-Slyked. It was believed that if the amino groups in this humin remained intact they should still give the nitrous acid reaction. The results are as follows:

	Humin nitrogen. mg.	Reactive with HNO_2. mg.
Tyrosine	2.360	2.45
Cystine	0.974	0.88
Tryptophane	13.820	1.90

From these results it must be concluded that in the case of tyrosine and cystine it was not the amino group that reacted with sugar to form humin but some other group, probably the (OH) in tyrosine and the (S-S) in the case of the cystine. If this were the case, then the cystine would presumably undergo reduction before reacting with the sugar.

In order to determine whether, as Gortner and Blish suggested, the furfurol obtained from sugar was responsible for the reaction, Experiments 54, 55, and 56 were performed as follows:

Experiment No.		Humin N. mg.	Per cent of total N.
54	0.2 gm. cystine + 2 cc. furfurol + 20 per cent HCl	7.00	32.0
55	0.2 gm. tyrosine + 2 cc. furfurol + 20 per cent HCl	8.40	55.0
56	0.2 gm. arginine + 2 cc. furfurol + 20 per cent HCl	12.75	21.5

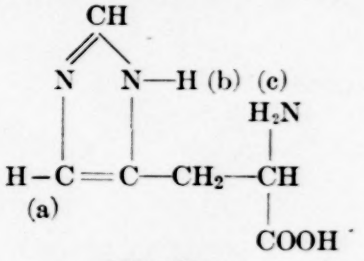
These results tend to show that the furfurol formed from sugars under the influence of acids may to a great extent be responsible for humin formation.

As to the effect of the different sugars on the reactive amino-acid, Experiments 20, 21, 22, 29, 38, and 46 show that xylose and fructose give higher results than glucose as a rule. This is to be expected, if it is admitted that furfurol or some other simple aldehyde is the active substance in these reactions.

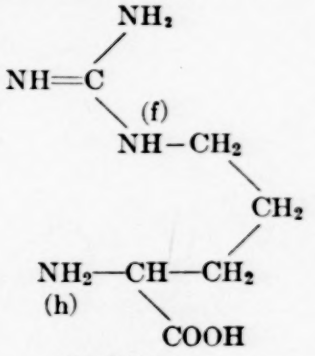
DISCUSSION OF RESULTS.

Some evidence is given which shows that the α-amino groups of arginine, histidine, and tryptophane take part in the reaction with sugars. On the other hand, the α-amino groups of alanine and leucine are unable to give the same reaction. Glutaminic acid and phenylalanine, although giving some humin nitrogen, likewise furnish no indication of reaction. At least for the present it may be admitted that the humin nitrogen in these cases—

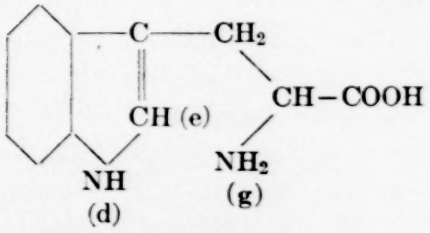
glutaminic acid and phenylalanine—was due to adsorption and not to a reaction. It was also shown that in tyrosine the reactive group is presumably the (OH) and surely not the α-NH_2. In cystine, as shown above, the α-amino group remained intact, so that presumably the reaction was with the mercaptan group. The question may then be asked: Why are the α-NH_2 groups of arginine, histidine and tryptophane more reactive than those of the other amino-acids? An attempt to explain this difference in behavior of the amino-acids towards carbohydrates, based on the present work and on some of the contributions reviewed in the first part of this paper, is here offered. It is generally stated that the properties of a compound are functions of its structure. It is, therefore, to the structure of these amino-acids that we must look for an explanation of their different behavior towards carbohydrates. The structural formulas of histidine, tryptophane, and arginine are given below.



Histidine.



Arginine.



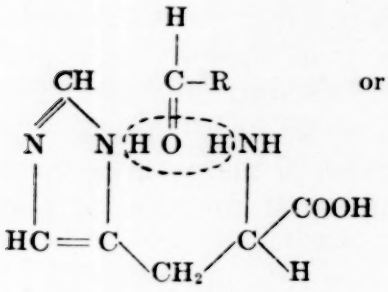
Tryptophane.

Several investigators have advanced the idea that humin formation is dependent on the presence of labile hydrogen in the amino-acid molecule (Samuelly, Grindley and Slater, etc.). Evidently judging from the results of the present work the two hydrogens of the α-amino groups of alanine and leucine are not

labile enough to give condensation products with carbohydrates at least under the conditions of these experiments.

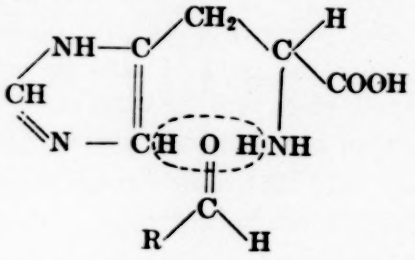
In histidine, arginine, and tryptophane, however, there are other labile hydrogens (a, b, c, d, e, f). The positions of these labile hydrogens with respect to the α-amino group are very favorable for ring formation. The reaction with a carbohydrate or furfurol may very well be thought of as taking place as follows:

Histidine.



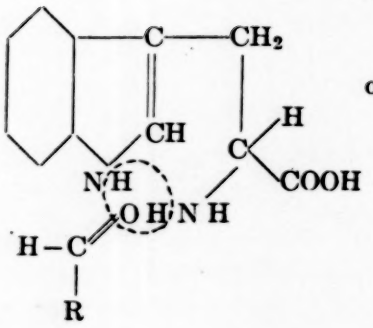
I

or



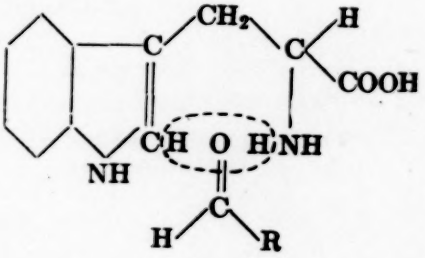
II

Tryptophane.



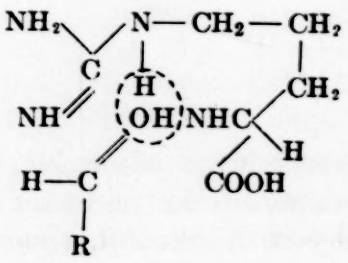
III

or



IV

Arginine.



V

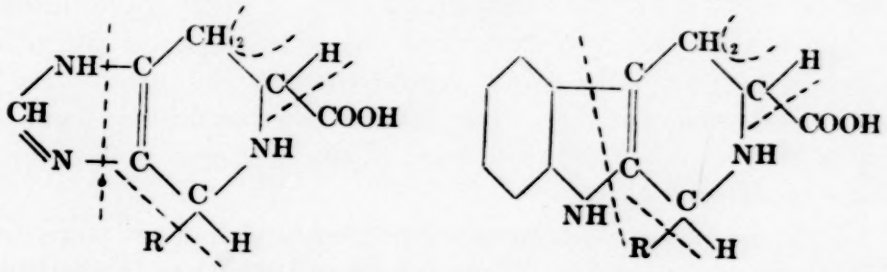
The following facts tend to support the idea of ring formation:

1. The intense color of the products.

2. Miss Homer[21] in her work on the condensation products of tryptophane with aldehydes, speaking of the action of glyoxal on this amino-acid, states:

> "Taking into consideration the necessity of the presence of an oxidizing agent and also the fact that the substance produced is intensely colored it is highly probable that in this reaction, besides the simple aldehyde condensation . . . . there has also been elimination of hydrogen accompanied by complex ring formation."

3. The fact that pyridine was obtained by Samuelly from his "melanoidins," was at one time used as an argument to indicate that a pyridine nucleus was found in proteins. This idea has been disposed of by Emil Fischer's work on proteins, but the fact remains that pyridine is found in the humin formed from proteins. This occurrence may be explained by Reactions II and IV thus:



4. The action of tyrosinase on tyrosine tends to support the idea of ring formation. Tyrosinase produces coloration with tryptophane but not with indol, skatol, or glycocoll. Therefore, the formation of the highly colored product requires the peculiar structure of tryptophane. This formation may be considered as taking place in the manner described above.

The differences in behavior between histidine, arginine, and tryptophane may again be referred back to the differences in their structure. Tryptophane being already a complex compound with a benzene and a pyrrol ring may form an insoluble four-ringed compound with furfurol, which is extremely resist-

[21] Homer, A., *Biochem. J.*, 1913, vii, 111.

ant to the action of acid. This will explain why tryptophane is converted into humin almost quantitatively. On the other hand furfurol may form with histidine and arginine products which are still more or less soluble and in the presence of strong acid may be hydrolyzed back to the free amino-acids. Thus as in the case of glutaminic acid, no formation of a ring compound takes place in strong hydrochloric acid solution. This view will explain why in weak acid or in aqueous solution both histidine and arginine react more readily to form colored products than in strong acid solution.

It is not claimed that the reaction given above gives the actual structure of the melanin molecule, since no evidence is available to indicate what happens to the rest of the molecule of the amino-acids during the reaction with sugars. This theory on humin formation is given here in the hope that it may serve as a guide for future work on the structure of these compounds.

No evidence was found in the present work to explain Samuelly's finding that when the humin obtained from sugar plus tyrosine was fused with alkalies, an odor of indol was obtained. It might have been possible that the tyrosine used contained traces of tryptophane which would explain the production of indol. Likewise the fact that pyrrol was obtained from his "melanoidins" can be traced back to the presence of the tryptophane nucleus in them.

Almost all of the experiments recorded in this paper were done with single amino-acids. There was found evidence (Experiment 31) to show that the reaction would be different, at least in the case of cystine and tyrosine, if other amino-acids were present in the reaction mixture with sugar. If cystine and proline were boiled together in the presence of glucose and 20 per cent HCl a larger amount of cystine nitrogen disappeared in humin formation than when cystine was boiled alone. The same was true when tyrosine and proline were boiled together. It would, therefore, be interesting to study the behavior of mixtures of different amino-acids when boiled with sugars, both in acid and aqueous solutions. Abderhalden and Guggenheim[19] working with tyrosinase along this same line already concluded that other amino-acids, when present, apparently take part in the production of the pigment.

CONCLUSIONS.

1. Alanine, leucine, phenylalanine, and glutaminic acid may be eliminated as important factors in humin formation, when subjected to the treatment used in these experiments. Proline, however, under certain conditions may be involved in humin formation.

2. The following amino-acids were responsible for humin formation, and in digestions, with 20 per cent HCl plus sugar, the proportion of their nitrogen disappearing was: Tyrosine, 15.0; cystine, 3.1; arginine, 2.33; lysine, 2.62; histidine, 1.84; tryptophane, 71.0 per cent.

3. Xylose and fructose were as a rule more reactive than glucose.

4. Arginine, histidine, and lysine reacted with sugars more readily in weak acid or aqueous, than in strong acid solutions.

5. Arginine, histidine, and tryptophane reacted with loss in reactivity of their amino nitrogen towards nitrous acid, but tyrosine and cystine reacted without any such loss.

6. A possible mode of reaction is suggested.

It is with pleasure that the writer acknowledges his obligation to Professor E. B. Hart, Chief of this Department, for giving him this problem, and for his many valuable suggestions during its execution.

THE DIRECT DETERMINATION OF UREA AND AMMONIA IN MUSCLE.

By JAMES B. SUMNER.

(From the Department of Physiology and Biochemistry, Medical College, Cornell University, Ithaca.)

(Received for publication, August 16, 1916.)

The determination of urea in blood and urine has been made both simple and accurate by the urease method of Marshall[1] as developed by Van Slyke and Cullen.[2] For some time the writer has had need of a suitable modification of this method in order to determine urea and ammonia in muscle and other tissue. Marshall[1] has successfully applied his urease method to the analysis of tissue, extracting with several portions of alcohol. The writer in collaboration with Fiske,[3] has used a similar method of extraction with alcohol, applying the method of Folin and Denis.[4] This procedure is apparently accurate but is tedious.

On account of the ease with which urea is capable of diffusing through cell membranes,[5] it appeared that the urease solution might be allowed to act directly upon the tissues containing urea, provided these tissues were ground finely, and enough time were allowed for the process of diffusion. At first the author tried grinding muscle with sand in a glass mortar, but gave up this procedure as unnecessary when it was found, a little later, that even coarsely chopped muscle tissue gave as high values for urea as samples that had been ground with sand to a very fine powder. That the urea quickly diffuses out of even rather coarsely chopped

[1] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 493. Marshall, E. K., Jr., and Davis, D. M., *ibid.*, 1914, xviii, 53.

[2] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211; 1916, xxiv, 117.

[3] Fiske, C. H., and Sumner, J. B., *J. Biol. Chem.*, 1914, xviii, 288.

[4] Folin, O., *J. Biol. Chem.*, 1912, xi, 507.

[5] Gryns, G., *Arch. ges. Physiol.*, 1896, lxiii, 86, and others.

muscle was shown by the fact that samples allowed to stand in urease solutions for ½ hour gave, upon aeration, no lower figures for urea than did samples that had been allowed to stand with urease for 1 and 2 hour intervals. This fact makes it possible to determine urea in muscle directly, and the writer sees no reason why it may not be determined in the same manner in any other tissue.

Van Slyke and Cullen have stated that the accuracy of the urease method is limited only by the error involved in titration, but I have found the greatest error to be in the process of aeration. The use of methyl red as indicator, of Jena glass, of sufficiently dilute acid and alkali solutions, of calibrated burettes, of a vertical plate glass mirror 2 feet back of the burettes as a means of avoiding parallax, and the precaution—as Van Slyke and Cullen advise—of keeping rubber stoppers and tubing from contact with strong alkalis and acids, make the process of titration an exceedingly accurate one.

EXPERIMENTAL PART.

Fresh muscle was obtained from cats and guinea pigs. They were etherized, killed by bleeding, and skinned; and the more accessible muscles removed with as little fat and fascia as possible. This muscle was minced in a "Universal" meat chopper. In order to obtain the tissue as homogeneous as possible, it was usually put through the meat chopper four or five times. The ground muscle was then transferred to a rubber-stoppered weighing bottle. A long glass spatula was found most useful in transferring samples of material to the bottoms of the large test-tubes (23 x 200 mm.) 9 cc. of water in portions were used to wash this spatula and to rinse down any bits of muscle that had stuck to the sides of the test-tubes. The most convenient weight of muscle to take as a sample was found to be from 2 to 3 gm. If larger amounts of muscle are used more water should be added to prevent the mass from becoming pasty. 1 cc. of a 5 per cent urease solution accurately made up was then added, the mixture well stirred with the end of the glass aeration tube, and after standing for ½ hour or longer the process of aeration was carried

out. For the determination of ammonia in muscle the mixture was stirred and aerated at once.

As the ground muscle was always found to be acid, no acid phosphate was added to it; and there was no danger from loss of ammonia.

The urease used was made by the method of Van Slyke and Cullen,[2] with the modification that less water was employed in the extraction of the urease, and a larger volume of acetone was used to precipitate it. The ammonia content of this dry preparation was carefully determined. New urease solutions were made up each day, and kept on ice. The author found it convenient to weigh out the urease into a large glass mortar and add the desired amount of water from a pipette, grind, and then transfer to a flask.

With the exception of the urease, all the reagents used were purified until almost absolutely free from ammonia. The potassium carbonate was made ammonia-free by boiling its solution to dryness, and heating until anhydrous.

For the liberation of ammonia, 5 gm. of anhydrous potassium carbonate were added to the solution, in which there were always present 10 cc. of water. The acid used for absorbing the ammonia set free was 0.01 N, and the alkali used in running back, 0.0088 N. In order to increase the height of the absorbing column of acid, 10 cc. of water were always added; this made a height in the test-tube of 6 to 7 cm., and under the conditions of the experiments allowed no measurable amount of ammonia to escape. With alkali even as dilute as 0.0088 N it was found that by using methyl red as indicator, and a standard end-point of sodium acetate and acetic acid, titrations could be run to within a very small fraction of a drop of alkali. The acid and alkali solutions were never allowed to come into contact with any but Jena or "nonsol" glass, with exception of the burettes which were well seasoned; Jena glass absorption tubes were used. Both aeration and absorption tubes were provided with eight small holes, as Van Slyke and Cullen advise. One drop of caprylic alcohol was added to the test-tube containing the acid and fifteen drops to the aerated mixture. The time of aeration was usually 40 minutes with an air current of 2 to 3 liters per minute, always run slowly for the first 3 minutes.

It was found that when the speed of aeration was 5 liters per minute, as advised by Van Slyke and Cullen, it was necessary to keep the test-

tubes containing the acid stoppered and to fit them with wide (8 mm.) bent tubes as traps to prevent loss of acid by spattering. Much more serious was the spattering of alkali from the test-tubes containing the aerated mixture. I found that no amount of caprylic alcohol would prevent the passing over into the acid of very slight amounts of fine spray. Although the alkali blown over with an air current of 5 liters per minute is very small in amount (usually 0.1 cc. of 0.01 N alkali with 20 minutes' aeration), this is not a negligible factor when 0.5 mg. or less of nitrogen is being dealt with. When the aeration is at a rate of not more than 3 liters per minute, this source of error is much lessened. The rate of aeration was measured by means of a Junkers gas meter and also by the method devised by Kober and Graves.[6]

Below are some results obtained by this method.

TABLE I.

Cat Muscle.

Weight.	Urease acted.	Mg. per 100 gm. moist muscle.	
		Urea and NH_3 N.	NH_3 N.
gm.	*hrs.*		
1.757	½	28.0*	
2.183	½	27.4*	
2.095	2	28.4	
1.811	2	28.6	
1.880	2	28.7	
1.177			10.4
1.486			10.0

* Once through chopper.

TABLE II.

Guinea Pig Muscle.

2.422	1	21.0	
2.476	1	20.6	
2.777	1	21.0	
2.760			14.6
3.165			14.1
2.521			15.3

[6] Kober, P. A., and Graves, S. S., *J. Am. Chem. Soc.*, 1913, xxxv, 1594.

TABLE III.

Guinea Pig Muscle.

3.190	1	26.8	
3.063	1	26.4	
2.587	1	26.1	
2.524	1	26.3	
3.246			13.0
2.591			13.9
	4 hrs. later.		
1.887	1	30.2	
2.727	1	30.1	
2.129			16.4
2.223			16.1

TABLE IV.

Cat Muscle.

Weight.	Urease acted.	Mg. per 100 gm. moist muscle.	
		Urea and NH_3 N.	NH_3 N.
gm.	*hrs.*		
2.515	$\frac{1}{2}$	24.9	
2.166	$\frac{1}{2}$	25.7	
2.560	$\frac{1}{2}$	24.8	
3.193	$\frac{1}{2}$	25.3	
2.632	2	24.7	
2.001			13.0
	4 hrs. later.		
2.990	$\frac{1}{2}$	24.6	
1.973	$\frac{1}{2}$	24.0	
2.154	2	25.0	
2.000			15.0

TABLE V.

Guinea Pig Muscle.

2.453	½	26.5	
2.695	½	27.0	
2.717	½	26.5	
2.590			12.8
2.259			13.0
	4 hrs. later.		
2.271	½	27.6	
2.176	½	28.0	
2.378	½	27.9	
2.316			13.8
2.310			13.8

TABLE VI.

Cat Muscle.

1.958	½	32.1	
2.266	½	32.7	
1.678	1	32.3	
2.266			12.6
2.217			12.1
	4 hrs. later.		
2.060	½	31.5	
1.975	½	31.2	
2.593	1	31.0	
2.302	1	31.4	
2.419			13.0
1.625			14.5

CONCLUSION.

It is noteworthy that the ammonia content of muscle should be so much higher than that of blood, the ammonia content of cat's blood being usually less than 1 mg. per 100 cc. Marshall and Davis[7] give figures for urea in the various tissues of dogs but make no mention of having made analyses for ammonia, or of having taken this into account.

[7] Marshall and Davis, *J. Biol. Chem.*, 1914, xviii, 53.

To avoid changes that might take place in chopped muscle, analyses were always started as quickly as possible, the urease being added usually within 40 minutes after the death of the animal. In order to observe whether any changes which would affect the figures for urea and ammonia did take place within the first few hours, I have, in a number of experiments, allowed the muscle to stand for a period of about 4 hours at room temperature (24–30°C.) and then carried out other analyses. The results show that there always occurs a slight increase in ammonia but do not allow any conclusion to be drawn with respect to the urea content except that its changes are inconsiderable.

The results published in this paper are believed to show that the determination of urea and ammonia in small samples of muscle can be carried out quickly and accurately by the method described.

THE CHYMASE OF SOLANUM ELAEAGNIFOLIUM.

A PRELIMINARY NOTE.

By A. BODANSKY.

(*From the Department of Physiology and Biochemistry, Medical College, Cornell University, Ithaca.*)

(Received for publication, July 27, 1916.)

In the fall of 1915 the writer's attention was called to the use by the Mexicans of New Mexico and Arizona of the berry of a certain weed as a substitute for rennet in the coagulation of milk. The plant was identified by Professor K. M. Wiegand, of the Department of Botany of the New York State College of Agriculture, as *Solanum elaeagnifolium* Cav.[1] The widespread occurrence of vegetable chymases[2] suggested the presence of a chymase in the berry of this plant, and the writer undertook to test the supposition.[3]

The entire berry of *Solanum elaeagnifolium* was used. It was collected in the late fall and was air-dry when received at the laboratory. After a few preliminary tests of the powdered berry and of its aqueous extract, which gave positive results, a more stable solid preparation was made as follows.

The berries were ground and the coarse powder obtained was extracted by percolation with a 5 per cent sodium chloride solution, containing a few drops of the essential oil of mustard, at a temperature of 5–10°C., after preliminary maceration for 24

[1] Wooton, E. O., and Standley, F. C., *Contrib. U. S. Nat. Herbarium*, 1915, xix, 573.

[2] Gerber, C., *Compt. rend. Acad.*, 1907–1913, and *Compt. rend. Soc. biol.*, 1907–1913; a summary of the investigations of vegetable chymases is contained in J. Effront's Les Catalyseurs biochimiques dans la vie et dans l'industrie, Paris, 1914, pp. 89 ff.

[3] The writer is indebted to Mr. L. E. Freudenthal, of Solomonville, Ariz., for the information which suggested the present work. The supply of the plant used in the preliminary experiments was obtained through the courtesy of Mr. Ph. Freudenthal.

hours at the same temperature. The liquid extract was clear, the first portions being brownish green in color, and the subsequent portions yellow. This extract was poured into thirty volumes of acetone, with constant shaking. The precipitate settled rapidly, leaving a clear supernatant liquid. The precipitate was transferred to a Buchner filter, sucked dry, then dried *in vacuo* over sulfuric acid. The powder obtained went easily into solution in water.[4]

In order to obtain comparable results in tests of the enzyme preparation, a milk of uniform composition was required. An artificial milk, similar to the one used by Blum and Fuld for testing gastric juice,[5] was prepared by mixing one part of powdered skimmed milk (Merrell-Soule) with nine parts of water, filtering the mixture through a thin layer of cotton, heating it to 80°C.-cooling in running water, and adding calcium chloride. In the earlier tests 0.5 cc. of a 20 per cent solution of calcium chloride was added to 100 cc. of artificial milk. The milk so prepared coagulated after a few minutes' boiling without the addition of enzyme, and was unsatisfactory for tests above 55°C. Satisfactory results were obtained by the use of 0.2 cc. of the 20 per cent calcium chloride solution in 100 cc. of artificial milk.[6]

The properties of the chymase of *Solanum elaeagnifolium* agreed, in general, with the properties of vegetable chymases described by Gerber.[2] It coagulated boiled natural milk without addition of calcium chloride. It proved more resistant to heat than animal rennin and obeyed the law of Segelcke-Storch within the lower range of temperature (up to about 55°C.).

[4] The method of extraction was essentially the same as that employed by Gerber (*Compt. rend. Soc. biol.*, 1909, lxvi, 890). The use of acetone to precipitate the mixture containing the enzyme is an adaptation of the method of D. D. Van Slyke and G. E. Cullen (*J. Biol. Chem.*, 1914, xix, 211) for the preparation of urease.

[5] Blum, L., and Fuld, E., *Berl. klin. Woch.*, 1905, xliv, 105, quoted by J. Effront (the original publication was not available).

[6] Blum and Fuld recommended the addition of 2 cc. of 20 per cent calcium chloride solution to 98 cc. of artificial milk. J. Wohlgemuth (Grundriss der Fermentmethoden, Berlin, 1913, p. 164), recommends the addition of 0.5 cc. of 20 per cent calcium chloride solution to 100 cc. of artificial milk. Gerber (*Compt. rend. Soc. biol.*, 1909, lxvi, 891) used boiled natural milk sensitized by the addition of 10 mg. molecules (1.11 gm.) of calcium chloride to the liter, performing his tests at 55°C.

In tests conducted between 37 and 55°C. the time of coagulation was inversely proportional to the quantity of the enzyme, other conditions being constant. Increase of temperature had the effect of increasing the rapidity of coagulation, but it also inactivated the enzyme. The optimum temperature was about 84°C., in a dilution of one part of the solid enzyme preparation to 20,000 parts of milk, coagulation taking place in about 1 minute. In a dilution of 1: 100,000 coagulation occurred in 10 minutes, the enzyme being obviously inactivated in the higher dilution by the longer exposure to heat.

Preliminary tests failed to discover any pronounced peptic or tryptic action of the extract. However, further tests will be necessary to justify a definite conclusion.

Work is in progress to determine the presence in *Solanum elaeagnifolium* of other enzymes than chymase, the distribution of the enzymes in the plant, the relative abundance and activity of the enzymes at various stages of growth, and the influence of various factors upon their action. The use of the berry by the Mexicans and the reported local use of *Galium verum* by cheesemakers of the West of England and of *Pinguicula vulgaris* by those of the Italian Alps[7] suggest the possibility of adapting the enzyme preparation of *Solanum elaeagnifolium* to the use by cheesemakers as a substitute for rennet extract. To investigate this possibility is one of the objects of the work outlined above.

The writer wishes to express his obligation to Professor J. B. Sumner, in whose laboratory this work was carried out, for his helpful suggestions and advice.

[7] Green, J. R., *Ann. Bot.*, 1893, vii, 112.

THE SEPARATE DETERMINATION OF CHOLESTEROL AND CHOLESTEROL ESTERS IN SMALL AMOUNTS OF BLOOD.

By W. R. BLOOR and ARTHUR KNUDSON.

(From the Laboratories of Biological Chemistry of the Harvard Medical School, Boston.)

(Received for publication, August 9, 1916.)

In the course of a study of the lipoids of human blood in normal and pathological conditions[1] it was found in almost all cases that cholesterol maintains a strikingly constant relation to the total fatty acids and to lecithin. Even in severe diabetic lipemia[2] where the amount of lipoids was many times the normal the relationship between cholesterol and the total fatty acids remained constant. The inference seemed justified that cholesterol takes an important part in fat metabolism. The most obvious way in which it would participate in fat metabolism is by its fatty acid esters which are always present in the blood and in most tissues, so that a knowledge of the balance between cholesterol and its esters in blood would be of great value in the study of this function. Such knowledge would also be of value in the study of other functions of cholesterol as, for example, the part which it plays as an antihemolytic[3] where the protective power depends on the amount of free cholesterol present. A method was sought which would permit the separate determination of cholesterol and its esters in small amounts of blood. The only successful means at present known for the separation of cholesterol from its esters is the precipitation of the cholesterol by digitonin as worked out by Windaus[4] and the following method is an adaptation of the digitonin precipitation to small amounts of material. It consists in (*a*) the determination of total cholesterol in an aliquot portion of an alcohol-ether extract of blood and (*b*) the

[1] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.

[2] Bloor, *J. Biol. Chem.*, 1916, xxvi, 417.

[3] Ransom, F., *Deutsch. med. Woch.*, 1901, xxvii, 194.

[4] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

determination of cholesterol esters in another aliquot after precipitation of the free cholesterol by digitonin. The difference between the two values represents free cholesterol.

The procedure is as follows:

1. Preparation of the Sample.—An alcohol-ether extract of 3 cc. of whole blood, plasma, or serum is made as described in the method for cholesterol already reported.[5]

2. Determination of Total Cholesterol.—This is made with an aliquot portion (generally 10 cc.) of the alcohol-ether extract according to the above method.[5]

3. Precipitation of Free Cholesterol and Determination of the Cholesterol Esters.—20 cc. of the alcohol-ether extract (or sufficient to contain about 0.5 mg. of combined cholesterol) are measured into a small flat-bottomed Erlenmeyer flask (50 cc.) and 1 cc. of 1 per cent alcoholic solution of digitonin is added. The whole solution is then evaporated just to dryness on the water bath or electric stove. The digitonin combines with the free cholesterol forming digitonin cholesteride while the cholesterol present as ester is not affected. The dried residue in the flask is then extracted by boiling out with successive small amounts of petroleum ether (boiling below 60°C.), filtering the extract through a plug of fat-free cotton in the stem of a small funnel.

In order to get a complete extraction with a small amount of solvent 15 cc. of the petroleum ether are first added, the flask is covered with a small watch glass (to prevent too rapid evaporation), and the whole boiled gently until about half the liquid is gone. The succeeding extractions are made in a similar manner with 7 to 8 cc. of petroleum ether. The petroleum ether dissolves the cholesterol esters but does not dissolve the digitonin precipitate. The combined extracts containing the cholesterol esters are then evaporated just to dryness, and the esters taken up with chloroform as in the method for total cholesterol.[5] The chloroform extracts, together slightly less than 5 cc., are collected in a 10 cc. glass-stoppered cylinder (previously calibrated), cooled, and made up to 5 cc. 5 cc. of a standard cholesterol solution in chloroform (containing 0.5 mg.) are measured into a similar 10 cc. cylinder. To each solution are added 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid, the cylinders

[5] Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.

stoppered, and the solutions mixed by inverting several times. They are then set away in the dark for 15 minutes after which they are transferred to the cups of the colorimeter and the readings made as usual, setting the standard at 15 mm. Artificial light (a 100 watt nitrogen-filled Tungsten light and "daylight" glass) was used throughout these determinations.

DISCUSSION AND RESULTS.

In working out the method for use the following points required to be determined.

a. Whether digitonin as used in the method gives a sufficiently complete precipitation of the cholesterol so that it could be used for the separation of the minute quantities of cholesterol involved in the determination.

b. Whether the solvent used dissolved the cholesterol esters without dissolving any measurable quantity of the precipitated digitonin cholesteride, or dissolving any other substance which would interfere with the determination.

c. Whether cholesterol esters give quantitative values for the cholesterol which they contain. Although it has been claimed[6] that such was the case it seemed desirable to test the point further.

Tests were made as follows:

(1) Portions of 0.5 to 1.5 mg. of cholesterol in alcohol ether were treated with digitonin according to the directions, the residue extracted, and examined for cholesterol by the colorimetric procedure. No color was found in any case so that the treatment was adequate for the complete isolation of free cholesterol.

The digitonin cholesteride, while insoluble in petroleum ether, was found to be measurably soluble in ether and still more in chloroform.

(2) 5 cc. portions of a solution of cholesterol palmitate containing 1.06 mg. of the ester, equivalent to 0.66 mg. of cholesterol, gave (*a*) by the method for determination of total cholesterol 0.681 and 0.687 mg., (*b*) by the method for determination of cholesterol esters 0.675, 0.681, and 0.681 mg.

(3) 5 cc. of the cholesterol palmitate solution to which was added 0.5 mg. of cholesterol gave by the ester method 0.680 and 0.680 mg. cholesterol.

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[6] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

(4) To 3 cc. of extract of blood plasma, found by the ester method to contain 0.441 mg. of cholesterol as ester, were added 3 cc. of the cholesterol palmitate solution containing 0.396 mg. of combined cholesterol. The whole sample then contained theoretically 0.837 mg. of combined cholesterol. Found: 0.815 mg.

(5) To three 5 cc. samples of plasma extract each containing 0.748 mg. of cholesterol as ester were added respectively 0.5, 1.0, and 1.5 mg. of cholesterol, and the esters then determined as usual. The results obtained were 0.742, 0.754, and 0.742 mg., respectively.

The following separations made on human plasma will show the possibilities of the method.

TABLE I.

Cholesterol and Cholesterol Esters in Blood Plasma (Mg. of Cholesterol per 100 Cc. of Plasma).

Sample.	Total cholesterol.	As ester.	Free.	
				Per cent of total.
Diabetes.....	538	245	293	54.5
		250	288	53.5
"	312	108	204	65.4
		111	201	64.4
"	508	215	293	57.7
		218	290	57.1
"	250	102	148	59.2
		100	150	59.9
"	556	358	198	35.6
		362	194	34.9
"	268	166	102	38.1
		168	100	37.3
"	182	116	66	36.3
		114	68	37.4
Normal.....	180	117	63	35.0
"	291	164	127	43.6
"	196	117	79	40.3
"	203	123	80	39.3

In a well planned investigation recently published[7] Mueller has questioned the correctness of the values for cholesterol obtained by colorimetric methods. He finds that while both colorimetric and gravimetric (precipitation with digitonin) methods give the same results with ordinary cholesterol the colorimetric methods give higher results with blood. He comes to the conclusion that there are other substances, probably however, closely related to cholesterol, present in blood which give the color reaction but which do not precipitate with digitonin and that therefore the gravimetric method gives more accurate results. He admits, however, that digitonin may precipitate part of these substances from blood and that the digitonin precipitate so obtained is somewhat soluble in the ether used for washing it (as has been found in the work above). Other points brought out in his investigation are a corroboration of the findings[5] that the alcohol-ether treatment as above performed gives a complete extraction of blood and that the results for cholesterol from this extract are higher than those obtained by other colorimetric methods.

The possibility that substances different from ordinary cholesterol but closely related to it may be present in blood has of course been realized, but in view of the lack of definite knowledge regarding their chemical nature, their behavior with digitonin and the Liebermann-Burchard reagent, or even of their presence, an extended discussion of their influence on the cholesterol determination does not seem advisable.

If, as Mueller claims, these substances give the color reaction but do not precipitate with digitonin (or to an insignificant extent) the values for cholesterol esters obtained by the procedure described above would be relatively higher, and those for free cholesterol relatively lower, since the error produced by these substances would fall on the esters with which they would be included if they were not precipitated by the digitonin. As a matter of fact the opposite appears to be the case, since, as may be seen by the values for normal plasma given in the table, and as will be shown more fully in a later publication, the values for free cholesterol are both relatively and absolutely *higher* than

[7] Mueller, J. H., *J. Biol. Chem.*, 1916, xxv, 549.

those reported in the literature.[8] Either there are no "other substances" in blood plasma or they behave, when treated with digitonin, in the same way as ordinary cholesterol.

The colorimetric methods are the only ones which can be used with small amounts of blood and which are therefore suitable for use in the extended study of conditions in living animals, including human beings. The procedure in these methods is relatively simple, the time required short, and the color a satisfactory one to measure. They give, moreover, higher values than the gravimetric method. Until the presence of these "other substances" is definitely shown and their chemical nature determined, the use of the colorimetric methods for the determination of cholesterol and its esters in blood seems fully justified.

[8] Mueller, *J. Biol. Chem.*, 1916, xxv, 561.

THE ISOLATION OF A GROWTH-PRODUCING SUBSTANCE FROM SHEEP PANCREAS.

By WALTER H. EDDY.

(*From the Chemical Laboratory of the New York Hospital.*)

(Received for publication, July 21, 1916.)

The following account covers the preliminary work done to establish the presence of a vitamine in the water-soluble portion of the alcohol extract of sheep pancreas. Experiments are now in progress to establish more fully its nature and properties.

The first attempt to demonstrate the growth-producing substance failed. The method followed was that recommended by Funk, as follows:

1,500 gm. of sheep pancreas were obtained from freshly killed sheep, minced with a meat chopper, and extracted with 4 liters of 95 per cent alcohol (to which was added enough hydrochloric acid to make the alcohol about 4 per cent HCl). The acid alcohol and pancreas were distributed in mason jars and shaken for 3 hours in the shaking machine. At the end of this period the alcohol extract was filtered off and evaporated nearly to dryness *in vacuo* at a temperature of 38°C. The final syrup was then evaporated to dryness before an electric fan at room temperature. (In all succeeding evaporations the fan method was used, the liquid being placed in shallow dishes and fanned with a continuous stream of air at room temperature.) The residue was then melted at 50°C. on a water bath and taken up with a liter of distilled water. This mixture was filtered first on a hot funnel maintained at 38–40°C. The filtered extract was again evaporated to dryness before the fan and the residue again taken up with *cold* distilled water, and filtered at room temperature. This final water solution was next made up to 5 per cent sulfuric acid strength and precipitated with a 50 per cent solution of phosphotungstic acid in 5 per cent sulfuric. In making this precipitate care was taken to avoid excess of phosphotungstic acid. The precipitate was then filtered off and washed thoroughly with 5 per cent sulfuric acid. After washing, it was mixed in a mortar with solid barium hydrate. After thorough mixing water was added and the entire mixture placed in a mason jar and shaken for 3 hours. At the end of this time the mixture was filtered and the filtrate carefully neutralized with sulfuric acid to remove the excess of baryta. After again filtering to remove the barium sulfate the liquid

was evaporated before the fan and the residue taken up in absolute alcohol to free it of inorganic salts. The alcohol solution was then precipitated by means of a saturated alcoholic solution of mercuric chloride. This precipitate was filtered off, suspended in water, and subjected to a stream of H_2S until all the mercury was converted into the sulfide. The latter was then removed and the mercury-free liquid treated with silver nitrate to precipitate the purines. These purines were then filtered off in turn and the filtrate which contained an excess of silver nitrate was precipitated with silver-baryta. This last was accomplished by first determining the presence of enough silver nitrate to yield a brown precipitate on the addition of a saturated solution of barium hydrate. This silver baryta precipitate was washed thoroughly to free it of NO_3 ions and then suspended in water and the silver converted into the sulfide with H_2S and filtered off. There remained a solution which was supposed to contain the vitamine more or less contaminated by the presence of Ba, SO_4, and Cl ions. These were carefully eliminated by use of sulfuric acid, barium chloride, carbon dioxide gas, and neutral moist silver oxide. The final purified solution was then used in the growth experiments.

Using the material, prepared by this method, the attempt was made to demonstrate its growth-inducing properties by its introduction into the basal diet of young male white mice. For a basal diet in these experiments and in the successful experiments with rats, Mendel's casein diet was used. Instead, however, of using highly purified reagents the diet was made up of the following:

	gm.
Casein (Merck's technical)	180
Starch (Duryea's corn starch)	325
Granulated sugar	170
Agar	50
Lard (Armour's)	250
Salt mixture	25

The salt mixture was composed as follows:

	gm.
$Ca_3(PO_4)_2$	10
K_2HPO_4	37
NaCl	20
Na citrate	15
Mg "	8
Fe "	2
Ca lactate	8

The ration was made into a paste by adding the melted lard to the dry ingredients and working in the agar after softening it with distilled water.

The animals were given distilled water to drink, each animal was allowed to eat all he wished, and the average daily consumption was noted. The reserve food was kept in the ice chest until needed and made up fresh at least once in 2 weeks. Fig. 1 indicates the typical results obtained from the fifteen mice used in this experiment.

The results of this experiment indicated clearly that the pancreas extract used contained no vitamine, or at least in too small quantity to produce growth results. The extract was given in 5 cc. doses and contained 0.0021 gm. of solid in each 5 cc. Later

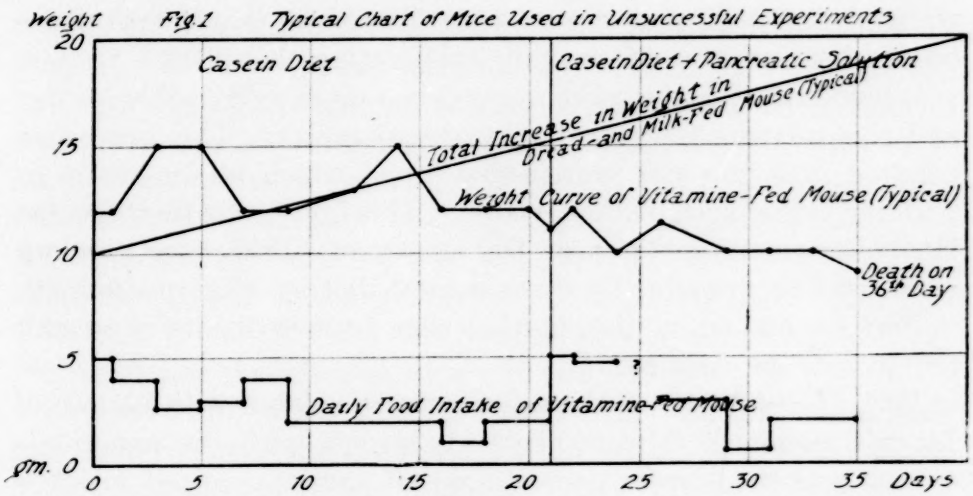
Weight
Fig 1 Typical Chart of Mice Used in Unsuccessful Experiments
20
15
10
5
gm. 0
Casein Diet
Casein Diet + Pancreatic Solution
Total Increase in Weight in Bread-and Milk-Fed Mouse (Typical)
Weight Curve of Vitamine-Fed Mouse (Typical)
Death on 36th Day
Daily Food Intake of Vitamine-Fed Mouse
0 5 10 15 20 25 30 35 Days

FIG. 1.

the extract was concentrated until 5 cc. contained 0.0123 gm. of solid, without favorable results. It contained only a trace of nitrogen.

Dr. Funk kindly checked the method outlined above and from the results we were led to believe that the failure to obtain the vitamine was due either to the loss of the material in the process of purification or to the small amount of material present. Dr. Funk had meanwhile been able to show that doses of yeast vitamine entirely adequate to cure beri-beri in pigeons were not of sufficient power to produce growth in rats. I therefore decided to repeat the experiments with a larger supply of material and to check the effect of the extract at each step of the purification. These latter experiments have now progressed sufficiently to

justify the claim that pancreas does contain the vitamine and in considerable amount. The results to date are given in the following report.

Demonstration of the Presence of Vitamine in Sheep Pancreas.

Materials Used.—3,620 gm. of minced sheep pancreas were extracted with 5,300 cc. of 95 per cent alcohol of 0.8 per cent HCl strength. After thorough extraction the alcohol extract was filtered off and evaporated to dryness before the fan at room temperature. The residue was taken up with distilled water, warmed to 38°C., and the mixture filtered at that temperature on the hot funnel. After again evaporating this filtrate to dryness before the fan the residue was taken up in cold distilled water and this mixture filtered at room temperature. This procedure resulted in a fat- and protein-free liquid which was made up to 2 liters volume with distilled water. This liquid was then divided into four equal portions of 500 cc. each. These four portions were used to prepare the experimental diet solutions as follows:

Part I.—500 cc. of the solution were kept in the ice chest and fed to rats as required.

Part II.—500 cc. of the solution were treated with 25 gm. of Lloyd's reagent.[1] The reagent was shaken with the pancreatic extract and the powder then filtered off and later mixed with the food of the rats. The filtrate was also retained and used in the diet of other rats as a control.

Part III.—In accordance with the method of Jacobs,[2] 500 cc. of the solution were made acid with hydrochloric acid to 5 per cent strength and then precipitated with a solution of 25 per cent phosphotungstic acid in 5 per cent HCl. After washing this precipitate with acid (5 per cent HCl) it was removed to a separatory funnel and into this funnel was put one volume each of amyl alcohol, ether, and water, made acid with HCl. Shaking this

[1] Seidell, A. (*U. S. Pub. Health Report*, No. 325, 1916), claims that Lloyd's reagent—colloidal hydrous aluminium silicate—exerts a selective adsorption for vitamine. His experiments were conducted with yeast vitamine.

[2] Jacobs, W. A., *J. Biol. Chem.*, 1912; xii, 429. See also Van Slyke, D. D., *ibid.*, 1915, xxii, 283.

mixture caused the phosphotungstic acid portion of the precipitate to pass into solution in the amyl alcohol and the rest of the precipitate passed into solution in the water. This water solution was then filtered off, carefully neutralized with NaOH, and used to feed certain rats.

Part IV.—500 cc. were held in reserve.

In the feeding experiments I had then for use the following solutions: A. A filtered water-soluble portion of the alcohol extract of pancreas. B. Lloyd's reagent after shaking with the water-soluble portion of the alcohol extract of pancreas and supposedly containing the adsorbed vitamine. C. Filtrate from the water-soluble portion of the alcohol extract after treatment with Lloyd's reagent. D. The phosphotungstic precipitate of the water-soluble portion of the alcohol extract of pancreas held in neutral water solution after freeing of phosphotungstic acid with amyl alcohol according to Jacobs' method. In the following descriptions these solutions will be designated by the letters used. All the liquids (A, C, and D) and the solid, B, were kept in the ice chest throughout the entire period of the experiment. This was done as a precaution and not because of any definite proof that the substance deteriorates at room temperature.

Demonstration Methods.—For these experiments young male rats (white) were used, ranging from 40 to 90 gm. in weight at the beginning of the experiment. They were kept in separate cages and the casein diet was used as the basal food. In order to make sure that the casein was vitamine-free, all that was used in the food of the rats was first boiled for several hours in 95 per cent alcohol. The food was placed in feeding boxes so arranged as to prevent contamination by feces. Distilled water was given for drinking purposes. Twelve rats were used in the feeding experiments. Four were maintained on a mixed diet of bread, milk, carrots, cabbage, etc., to determine normal growth. Three of these are plotted in Fig. 2 (Rats 6, 7, and 12). One of the four developed scurvy and his curve is not included.

Rats 1 and 2 were fed the casein diet for a period of 16 days. In this time they steadily declined in weight, as shown by the heavy line. (The dotted line shows the average food intake per day.) On the 16th day the casein diet was mixed with 16 cc. of the water-soluble pancreatic extract A. At each succeed-

ing weighing period new food was given mixed with 10 cc. doses of the extract. The upward rise in the weight curve was immediate and was steadily maintained throughout the entire experimental period of 23 days following the first dose. These results

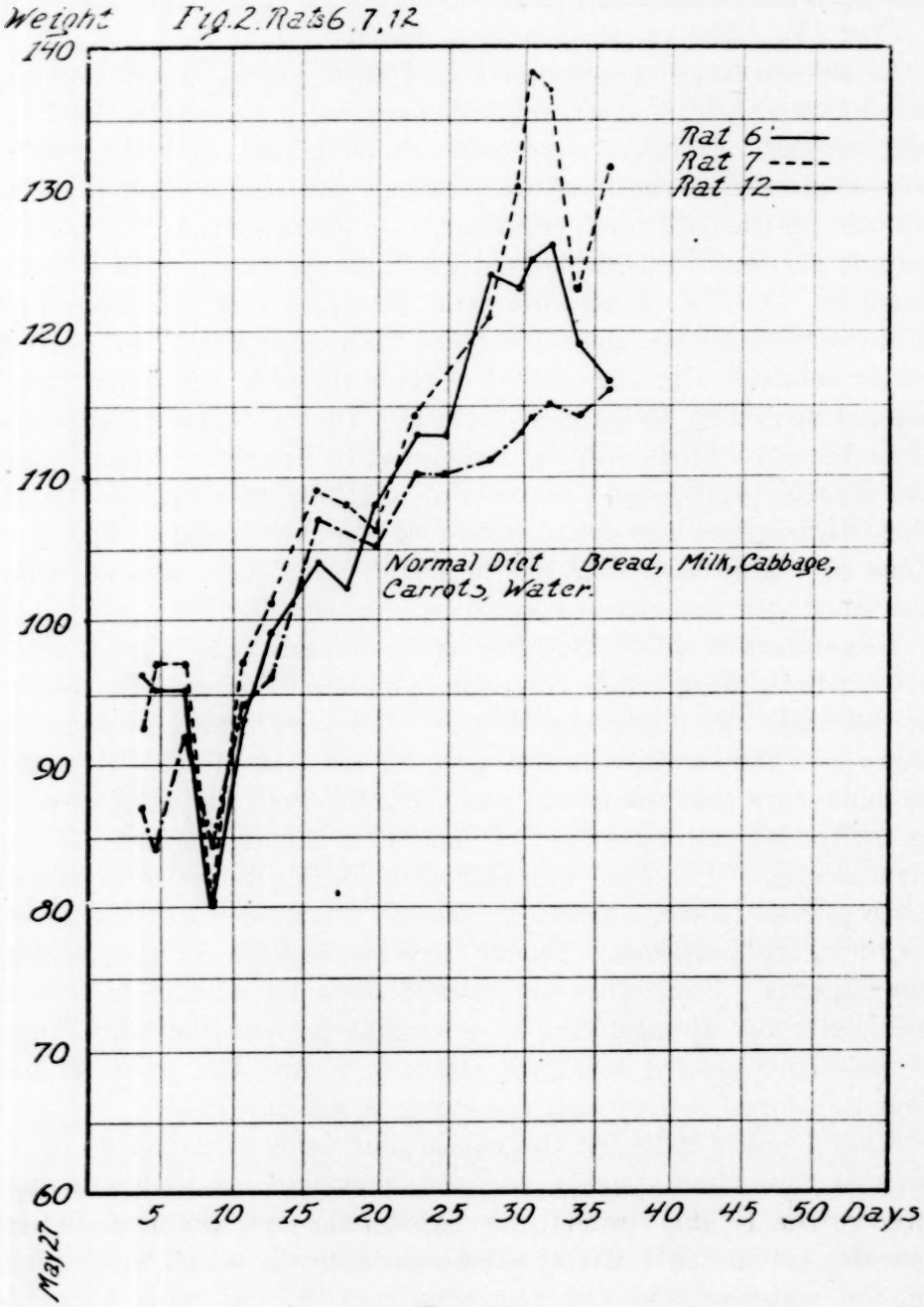

FIG. 2.

indicated clearly the power of the extract to induce growth and also to develop increase in daily food consumption.

As in the case of Rats 1 and 2, the diet of Rat 3 was of the casein

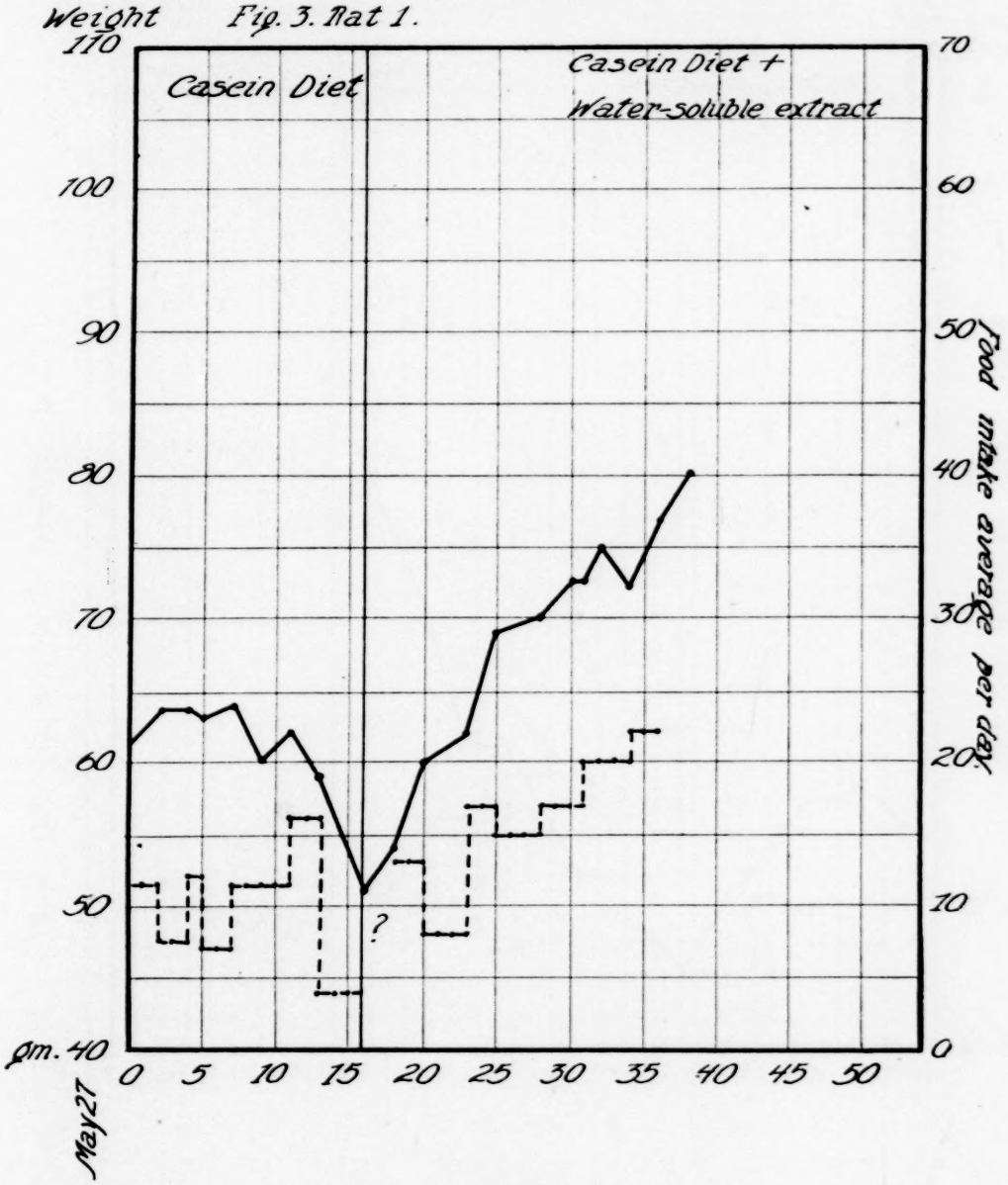

FIG. 3.

mixture for 16 days with bare maintenance of weight for that period. On the 16th day the water-soluble extract A was added and the corresponding rise in weight occurred. On the 25th

day the rat was put back on the casein diet and in the next 6 days declined 6 gm. in weight, showing that the presence of the extract was necessary to maintain growth. On the 32nd day

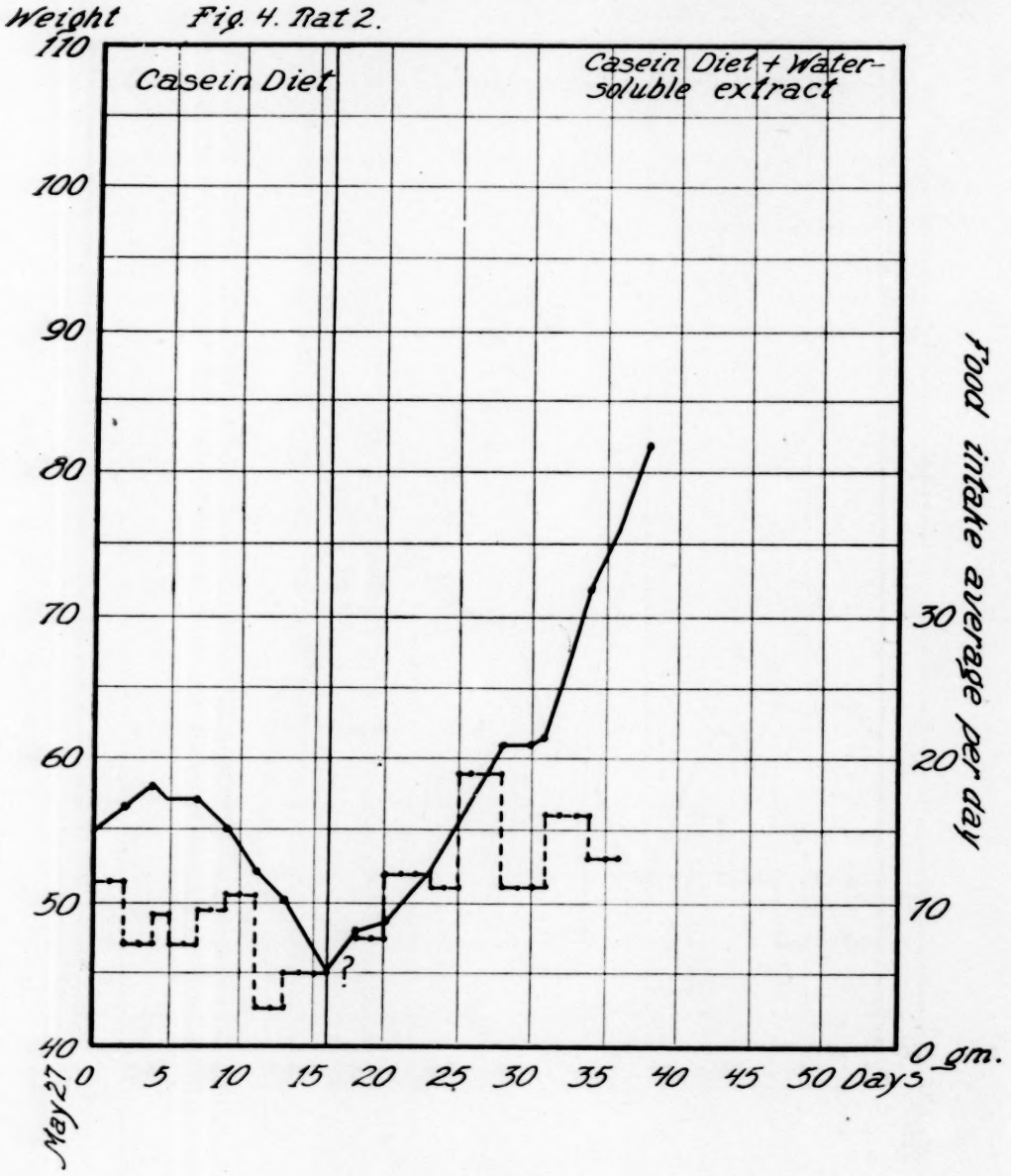

FIG. 4.

this rat was given a dosage of 10 cc. of the phosphotungstic precipitate D. The animal immediately began to gain weight and continued to do so to the end of the experiment on the 39th

day. This experiment demonstrated that the growth-producing power of the water extract was not lost in the first separation step.

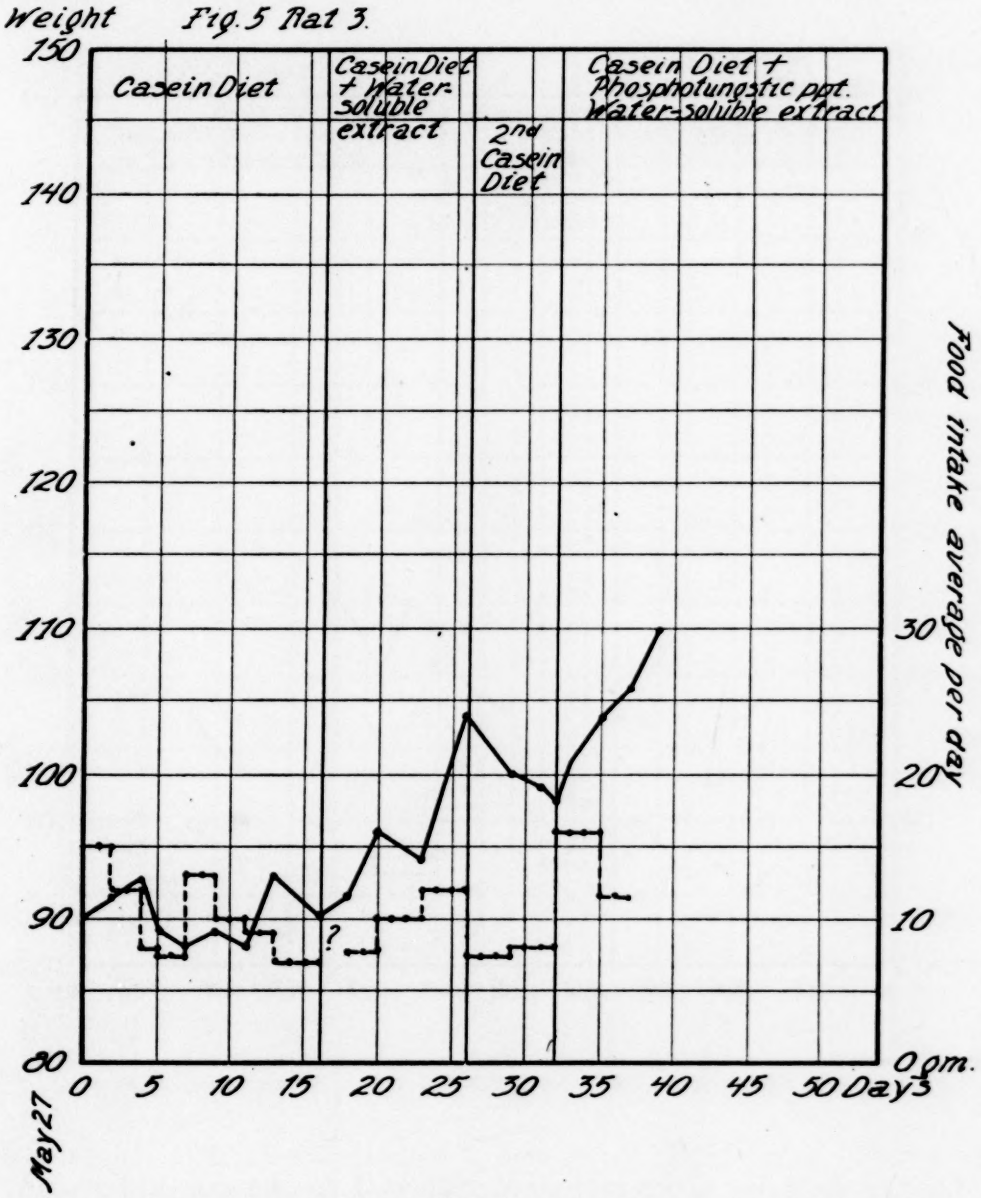

FIG. 5.

Rats 4 and 5 were given 18 days of casein diet with maintenance or decline. On the 18th day 12 gm. of Lloyd's reagent, B, were added to the diet. At each succeeding period 5 to 6 gm.

were added. In the 10 days of this treatment the rats showed an increase in weight, demonstrating the power of this substance to adsorb the vitamine and confirming Seidell's[1] conclusions. (Further confirmation of this point was given by Rat 9.)

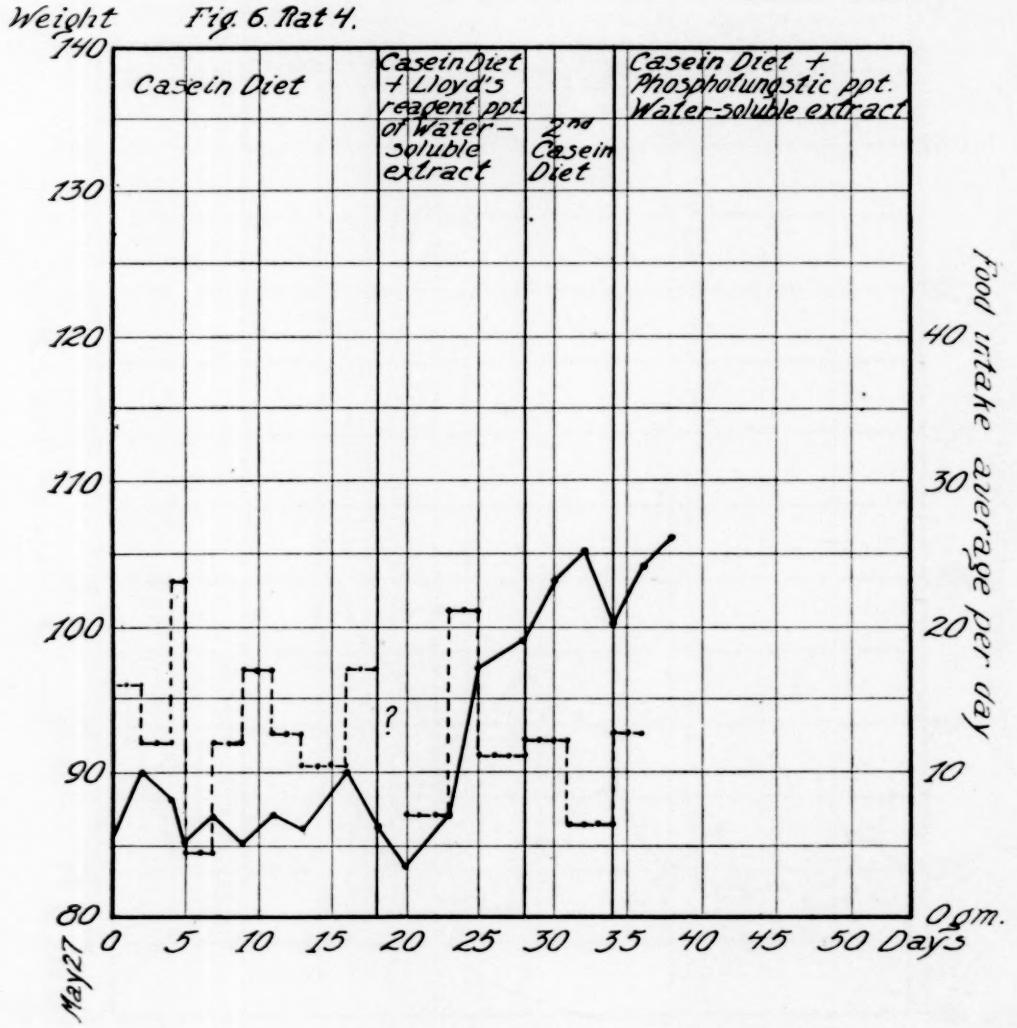

FIG. 6.

On the 28th day these rats were returned to the casein diet and it was interesting to note that Lloyd's reagent was found to persist in its stimulating effects for some 3 or 4 days after its feeding had stopped. At the end of 6 days the rats had begun to decline. Both of these rats were made to resume their up-

ward rise in weight by the addition of 10 cc. doses of the phosphotungstic precipitate D to the diets on the 32nd and 34th days respectively, further confirming the results with Rat 3.

Rat 9 was fed casein diet for 23 days with a marked decline

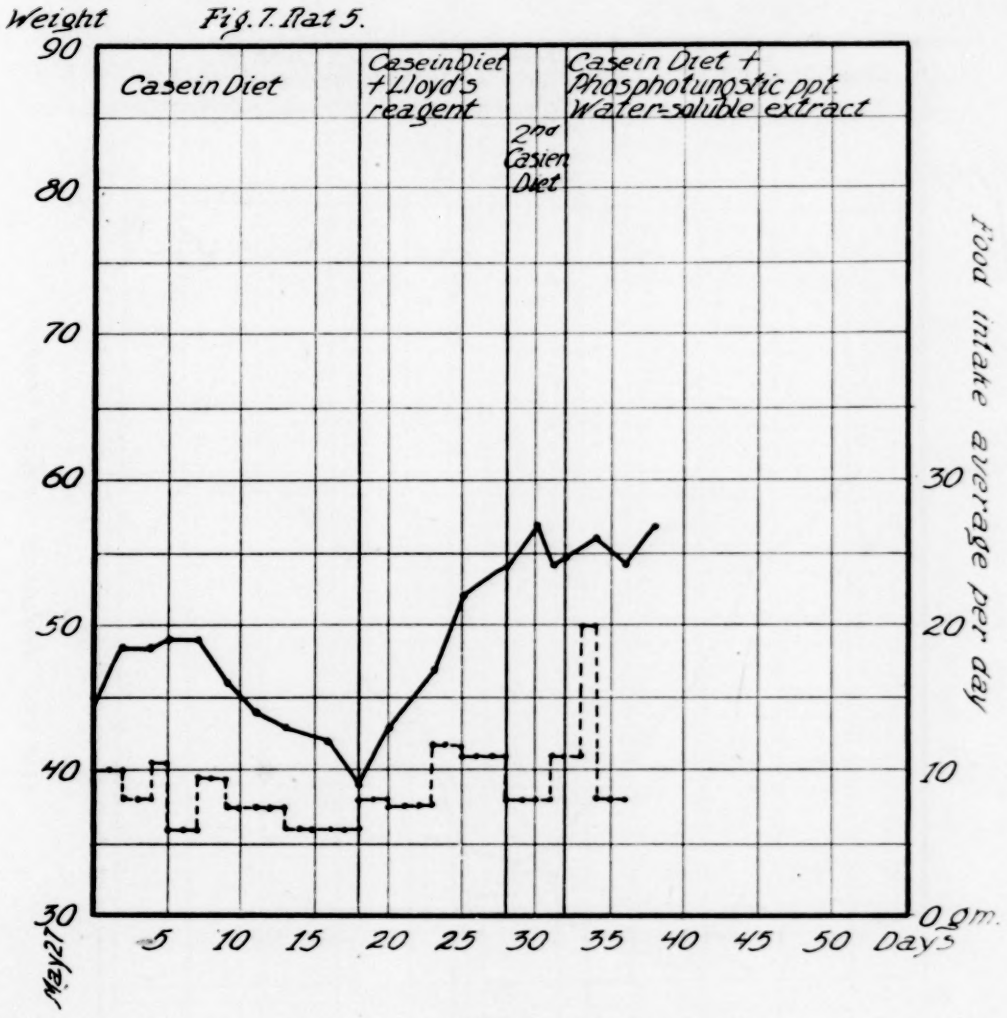

FIG. 7.

in weight. On the 23rd day a dosage of the filtrate C from Lloyd's reagent was begun (10 cc. amounts at each weighing period). This failed to do more than produce a lessening in the decline of the curve, indicating that the Lloyd reagent removes at least the greater part of the vitamine. At the end of 8 days of this diet the rat was given 1 day's ration of casein food alone

and then a dosage of the phosphotungstic precipitate was begun (D). Recovery and increase were immediate.

Rat 10 was fed casein diet for 32 days with a decrease in weight of some 30 gm. In spite of this long period of decline the feed-

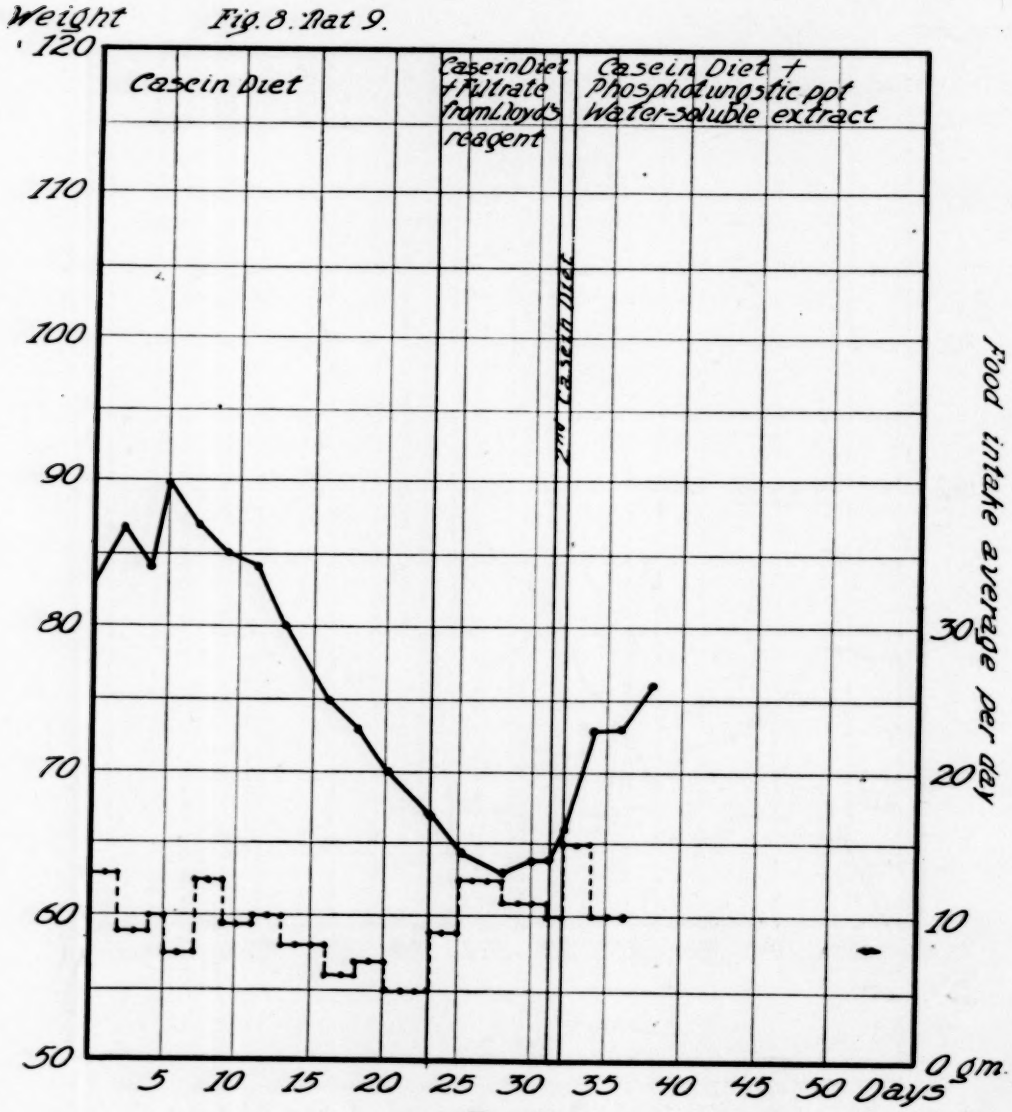

FIG. 8.

ing of the phosphotungstic precipitate D on the 32nd day brought about immediate rise in weight which was maintained.

At the end of the charted period the rats were all in good condition except the one control rat mentioned that showed incipient

scurvy. None of the extract-fed rats showed any signs of scurvy or of other dietary diseases.

GENERAL CONCLUSIONS.

The results of the rat experiments seemed to prove conclusively that the water-soluble portion of the alcoholic extract of pancreas contains a substance that is capable of inducing marked increase in growth. This substance is removed from the extract

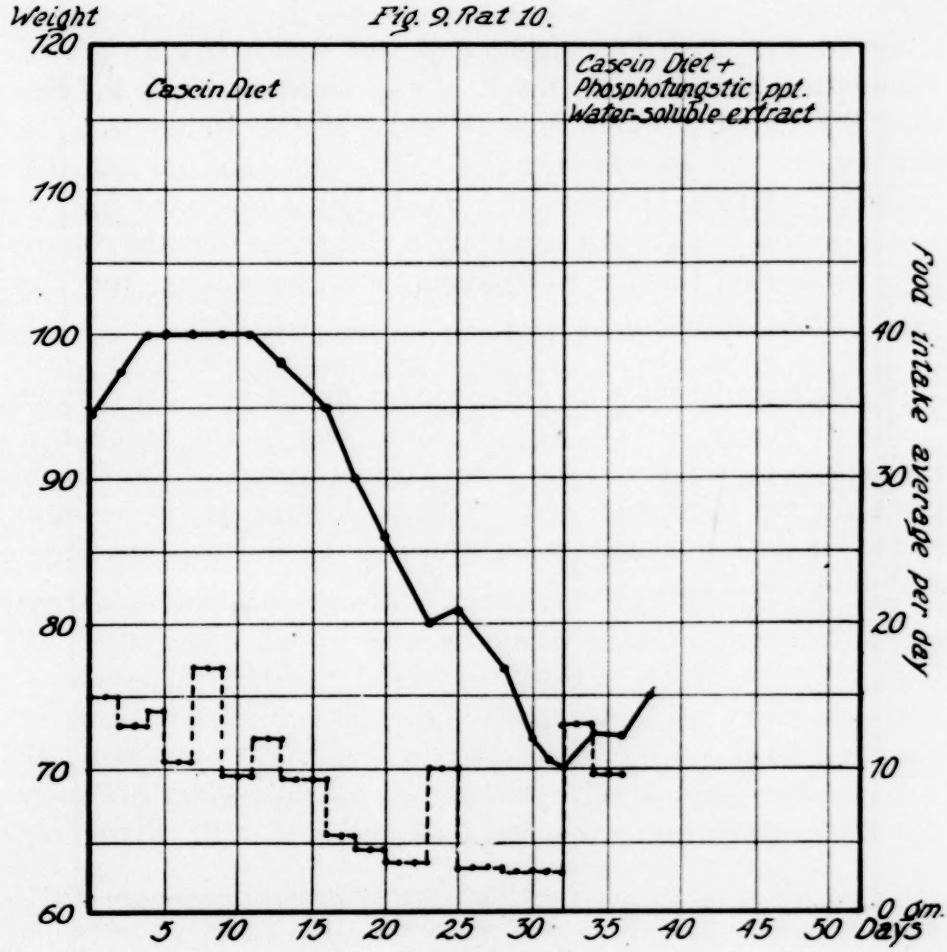

FIG. 9.

without loss of power by treatment with Lloyd's reagent. It is also precipitated in the phosphotungstic precipitate of the extract. It is not a protein or fatty substance. It occurred to me that the results might indicate not a vitamine but a happy

combination of amino-acids. To determine this point I secured amino nitrogen determinations on solutions A, C, and D. These solutions were analyzed by Dr. J. C. Bock of Cornell Medical College, to whom they were given as lettered samples. The results of the analyses were as follows:

Solution A contained 4.839 mg. of amino N per cc.
" C " 4.854 " " " " " " "
" D " 1.370 " " " " " " "

Solution C was the solution from which the vitamine was removed with Lloyd's reagent and had practically no growth-stimulating power. The result of the above analysis indicates that the Lloyd powder removed none of the amino-acids, yet did remove the growth stimulus. The fact that the phosphotungstic solution D contained much less amino-acid and yet was powerful in a growth stimulation also tends to make the conclusion stronger that we were dealing with something other than amino-acid stimulus.

In conclusion I wish to acknowledge my indebtedness to Dr. Nellis B. Foster, to Dr. Wm. J. Elser, and to Dr. Stillman for the facilities given me at the New York Hospital, to Dr. Casimir Funk for his suggestion and criticism throughout the research, and to Dr. J. C. Bock for his assistance in the matter of the amino-acid determinations.

STUDIES IN CREATINE METABOLISM.*

I. POSSIBLE INTERRELATIONS BETWEEN ACIDOSIS AND CREATINE ELIMINATION.

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(Received for publication, July 31, 1916.)

Current views[1] associate the elimination of creatine with some perversion of carbohydrate metabolism. The probability of a close relationship of this sort is indicated by the well known fact that a deficiency of carbohydrate in the body, as in starvation, leads to creatine elimination which may be checked promptly by ingestion of carbohydrate. Depletion of the body of carbohydrate may also be accomplished through the action of various toxic substances like phlorhizin, phosphorus, hydrazine, etc., or through disease as in diabetes. In each of the enumerated instances of carbohydrate deficiency, creatine appears in the urine. There are, however, experimental facts which the familiar hypothesis fails to explain. McCollum and Steenbock[2] found that in the pig a diet of corn products, among others, led to the appearance of relatively large quantities of creatine in the urine. The authors point out that the "character of the proteins in the diet appears to determine whether a large or a small creatine production takes place in the tissues. These observations leave little doubt that creatine may come from exogenous as well

* The results of this investigation were reported to the Society for Experimental Biology and Medicine, March 15, 1916. See *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 113.

[1] Recent literature concerning creatine may be found in the papers of Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213; Riesser, O., *Z. physiol. Chem.*, 1913, lxxxvi, 415; Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xiv, 9; 1913, xv, 283, 285; 1913–14, xvi, 169; 1915, xxi, 377, 383, 389. Benedict, S. R., and Osterberg, E., *ibid.*, 1914, xviii, 195.

[2] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912–13, xiii, 209.

as endogenous protein metabolism." Somewhat similar experiments of Folin[3] with oat feeding yielded comparable results. The dietaries employed, especially those of McCollum and Steenbock, cannot be regarded as lacking in carbohydrate, for in the creatine-producing diet was included a significant quantity of added starch.

Deficiency of carbohydrate usually means an accompanying acidosis, not necessarily caused by ketogenic substances, which presumably involves the tissues associated with creatine-creatinine metabolism. At any rate nearly every instance in which there is creatine in the urine is accompanied by an acidosis—generally a ketonuria also. The term acidosis in the sense used here means a disturbance in the acid-base equilibrium in the body, whereby the excessive production of acid or introduction of substances forming an acid ash causes the withdrawal of base from the body. In man it is probable that in general the body neutralizes such acid with ammonia, the neutralization of acid with other bases never having been demonstrated. Carnivora undoubtedly neutralize acids in a similar manner. With the rabbit, however, the neutralization of acid may not necessarily be performed by ammonia, for in experimental acid poisoning in these animals there is not a close relationship between the output of ammonia and the introduction of acid, but the other bases as sodium, potassium, calcium, and magnesium, are much increased in the urine. With the dog, on the other hand, such a relationship obtains and the other alkali bases are relatively little increased. The older explanation of these facts was to the effect that the herbivorous animal ingested too little protein material to furnish sufficient ammonia for neutralization purposes. Another possible viewpoint is that owing to the general character of the diet consumed by the herbivora a great excess of base is introduced into the body, as indicated by the general alkalinity of the urine of herbivora, which may be readily available for neutralization purposes under ordinary conditions. The store of such available base must be somewhat limited, however, since the great susceptibility of the rabbit to acid is well known. In view of this fact it would seem that in questions

[3] Reported at the meeting of the American Society of Biological Chemists, December, 1915.

involving the influence of acid upon varying phases of metabolic activity the rabbit would serve as an ideal experimental animal, much more so than the dog, which is very resistant to acid introduction.

The fact that in nearly every instance in which creatine appears in the urine there is an accompanying acidosis suggests the hypothesis that a condition of acidosis in the body is responsible for the appearance of creatine in the urine. To test it the following questions demand an answer. 1. If acidosis is induced, will creatine appear in the urine, even in the presence of an adequate carbohydrate supply? 2. Will the elimination of creatine disappear if the acidosis is abolished, quite independently of the factor of carbohydrate supply?

In the present paper a condition of acidosis was considered indicated when the urine of the rabbit became strongly acid, as shown by determination of the hydrogen ion concentration, employing sodium alizarine sulfonate as the indicator.[4] In the sense of the term acidosis as used here, that is, a condition of alkali depletion, the assumption given above is undoubtedly correct since ordinarily the rabbit secretes urine which is strongly alkaline.

The general plan of the investigation was as follows: Rabbits were fed upon diets containing adequate carbohydrate supply but of such a nature that one diet would yield a pronounced acid ash, another a distinctly basic ash, and finally the third type of diet consisted of a mixture of the other two. The acid-producing foods were whole oats and cracked corn, the mixture being designated the "grain diet." Fresh carrots constituted the base-producing food, designated "carrot diet." A dietary containing oats, corn, and carrots is called the "mixed diet."

The results obtained are to be found in Tables I to VI. Upon a diet of oats and corn containing an adequate supply of carbohydrate, creatine[5] promptly appears in the urine. A

[4] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393. The presence of phosphates in the alkaline urines interfered with accurate determinations of the hydrogen ion concentration; hence the figures given for the alkaline urines are only approximations.

[5] 24 hour specimens of urine were obtained by compression of the bladder through the abdominal wall. Creatinine was estimated according to the method of Folin and creatine by the procedure of Benedict and Myers.

markedly increased hydrogen ion concentration of the urine is always associated with this phenomenon. The presence of creatine in the urine is just as easily manifested upon a grain diet whether the latter is given to animals fresh from stock or after a relatively prolonged period in which the base-producing foods have predominated. Upon the grain diet the urine of the two rabbits tested, Tables VI and VII, acquired distinct tests for protein. The condition of albuminuria persisted throughout the period of observation. It is of considerable significance in the etiology of nephritis that *protein may appear in the urine as a result of a change in the character of the diet.*

Upon a carrot diet urinary creatine rapidly disappears as the urine becomes alkaline; or if creatine was originally absent from the urine it remains so.

Under the conditions outlined above the facts with regard to creatine elimination may be interpreted in two ways, first, the appearance of creatine in the urine on the grain diet and its disappearance on the carrot diet may be ascribed to the character of the proteins constituting the dietaries, or second, the phenomenon under discussion may be explained on the basis of the acid-base-producing potentialities of the dietaries. To determine the responsible factor animals were fed upon the "mixed diet" constituted by a mixture of the two types of dietaries. From the tables it is obvious that the protein *per se* in the diet is without special significance for upon the mixed diet creatine fails to appear in the urine and the reaction of the latter remains alkaline. The acid-producing power of the grain diet must therefore be considered as the potent factor in the production of creatinuria under the experimental conditions. To demonstrate still further the validity of this inference rabbits, maintained upon a "mixed diet," hence consequently without creatinuria and with alkaline urines, were given relatively small doses of hydrochloric acid by means of the stomach sound twice daily. This addition of hydrochloric acid to the "mixed diet" without change of proteins causes the appearance in the urine of significant quantities of creatine. Simultaneously the hydrogen ion concentration of the urine is markedly increased.

The facts enumerated above also confirm the findings of McCollum and Steenbock that creatine may appear in the urine

even when adequate carbohydrate is supplied; and they lead to the conclusion that creatinuria is not necessarily related to carbohydrate deficiency in the body. It would appear to be associated with a condition of acidosis which may and usually does accompany those states of metabolism in which carbohydrate deficiency is especially noticeable.

In general, the figures for creatinine are fairly constant for each individual animal, but an inspection of the tables will show that there is on the average a decided tendency for creatinine to be increased on the carrot diet and to be decreased when hydrochloric acid is added to the mixed diet.

The tables follow.

TABLE I.

The Relation of the Character of the Food Intake to P_H of the Urine and to the Elimination of Creatine.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Jan. 24	90	1.025	135	0	8.70	White male rabbit of 2,400 gm.
" 25	65	1.023	92	18	6.70	
" 26	100	1.030	72	45	6.30	Grain diet { 50 gm. oats. 50 " cracked corn.
" 27	60	1.035	90	34	5.30	
" 28	30	1.040	81	27	4.90	
" 29	50	1.030	81	31	4.70	Feces soft.
" 30	60	1.025	81	39	4.70	" "
" 31	60	1.024	77	39	4.70	" "
Average			**88**			
Feb. 1	60	1.030	81	13	5.30	Carrot diet: 300 or 500 gm. carrots. For first 2 days 300 gm. carrots were given. From this period on the animal received 500 gm. carrots.
" 2	300	1.010	90	5	7.38	
" 3	300	1.010	81	0	7.48	
" 4	300	1.010	93	0	7.48	
" 5	425	1.012	91	0	7.48	
" 6	275	1.025	92	0	8.0	
" 7	300	1.016	100	0	9.27	
" 8	350	1.015	90	0	8.70	
Average			**90**			
Feb. 9	250	1.020	73	0	8.70	Mixed diet { 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 10	120	1.015	88	0	8.70	
" 11	120	1.015	90	0	8.70	
Average			**83**			
Feb. 12	170	1.012	57	0	8.0	12 m. 50 cc. 0.25 N HCl by stomach tube.
" 13	130	1.010	66	10	4.90	{ 11 a.m. 50 cc. 0.25 N HCl by stomach tube. 6 p.m. 50 cc. 0.25 N HCl by stomach tube.
" 14	150	1.020	55	45	6.00	{ 11 a.m. 50 cc. 0.25 N HCl by stomach tube. 6 p.m. 50 cc. 0.25 N HCl by stomach tube.
Average			**59**			

TABLE II.

The Relation of the Character of the Food Intake to P_H of the Urine and to the Elimination of Creatine.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Jan. 24	50	1.038	95	0	8.70	Male rabbit of 2,200 gm.
" 25	60	1.035	95	11	6.70	
" 26	50	1.032	83	51	6.30	
" 27	50	1.030	101	39	5.30	Grain diet: 50 gm. oats. 50 " cracked corn.
" 28	40	1.035	81	36	5.70	
" 29	50	1.035	108	45	4.90	
" 30	50	1.035	81	45	4.70	
" 31	55	1.032	81	39	4.90	
Feb. 1	50	1.035	81	57	4.70	Urine showed distinct test for protein.
" 2	50	1.035	90	41	4.70	Previous to experiment urine was protein-free.
Average			**89**			
Feb. 3	110	1.020	97	31	4.90	Carrot diet for first 2 days 300 gm.
" 4	400	1.015	116	18	7.48	Carrot diet for remainder of period, 500 gm.
" 5	425	1.012	112	0	7.48	
" 6	275	1.025	104	0	8.0	
" 7	400	1.012	98	0	8.70	
" 8	350	1.015	113	0	8.70	Urine still shows traces of protein.
Average			**106**			
Feb. 9	170	1.020	94	0	8.70	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 10	100	1.025	70	0	8.70	
" 11	120	1.020	88	0	8.70	
Average			**84**			
Feb. 12	150	1.012	71	14	6.30	12 m. 50 cc. 0.25 N HCl by stomach tube.
" 13	110	1.020	68	68	4.90	11 a.m. 50 cc. 0.25 N HCl by stomach tube. 6 p.m. 50 cc. 0.25 N HCl by stomach tube.
" 14						Animal killed because of injury.
Average			**69**			

TABLE III.

The Relation of the Character of the Food Intake to P_H of the Urine and to the Elimination of Creatine.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Jan. 24	60	1.035	95	0	8.70	Male rabbit of 2,200 gm.
" 25	50	1.030	90	0	6.90	
" 26	90	1.030	109	53	6.00	
" 27	60	1.030	85	52	4.90	
" 28	60	1.025	112	27	4.90	Grain diet: 50 gm. oats. 50 " cracked corn.
" 29	70	1.025	81	63	4.70	
" 30	60	1.030	81	49	4.70	
" 31	70	1.025	81	57	4.70	
Feb. 1	70	1.025	81	63	4.70	
" 2	60	1.030	90	52	4.70	Urine previously protein-free contained distinct traces of protein.
Average			**90**			
Feb. 3	150	1.015	95	57	5.70	Carrot diet for first 2 days, 300 gm.
" 4	470	1.015	139	10	7.38	Carrot diet for remainder of period, 500 gm.
" 5	400	1.012	112	0	7.48	
" 6	375	1.015	131	0	8.0	
" 7	400	1.012	114	0	8.70	
" 8	375	1.015	120	0	8.70	Urine still contains protein.
Average			**118**			
Feb. 9	190	1.018	118	0	8.70	
" 10	120	1.030	97	0	8.70	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 11	170	1.015	100	0	8.70	
" 12	250	1.010	115	0	8.0	
Average			**107**			
Feb. 13	120	1.015	97	49	4.90	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 14	170	1.015	93	70	4.90	
" 15	160	1.015	92	43	4.70	
Average			**94**			
Feb. 16	425	1.015	100	13	9.27	
" 17	425	1.012	172	0	9.27	
" 18	350	1.015	125	0	9.27	Carrot diet: 500 gm. carrots.
" 19	400	1.015	134	0	9.27	
" 20	250	1.020	113	0	9.27	
" 21	120	1.035	114	0	9.27	
Average			**126**			

TABLE III.—*Concluded.*

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Feb. 22	275	1.015	113	23	7.38	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 23	120	1.025	100	72	5.70	
" 24	175	1.015	100	34	5.30	
Average			**104**			
Feb. 25	350	1.010	133	28	9.27	
" 26	465	1.013	128	0	9.27	Carrot diet: 500 gm. carrots.
" 27	325	1.017	114	0	9.27	
Average			**125**			
Feb. 28	80	1.035	136	0	6.90	
" 29	70	1.035	103	17	4.70	
Mar. 1	96	1.020	110	34	4.70	Grain diet: 50 gm. oats. 50 " cracked corn.
" 2	90	1.015	121	34	4.70	
" 3	93	1.020	126	14	4.70	
Average			**119**			
Mar. 4	265	1.010	130	0	9.27	Carrot diet: 500 gm. carrots.
" 5	430	1.015	138	0	9.27	Body weight at end of experiment, 2,280 gm.
Average			**134**			

TABLE IV.

The Relation of the Character of the Food Intake to P_H of the Urine and to the Elimination of Creatine.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Feb. 16	325	1.017	132	36	9.27	Male rabbit of 2,700 gm. Previous diet unknown.
" 17	350	1.015	123	14	9.27	
" 18	325	1.015	104	0	9.27	Carrot diet: 500 gm. carrots.
Average			**119**			
Feb. 19	400	1.015	134	0	9.27	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 20	140	1.025	113	0	9.27	
" 21	90	1.038	121	0	9.27	
Average			**123**			
Feb. 22	235	1.015	126	0	7.38	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 23	275	1.012	99	98	5.30	
" 24	235	1.015	118	24	5.30	
Average			**114**			
Feb. 25	275	1.015	115	16	9.27	
" 26	310	1.015	119	7	9.27	Carrot diet: 500 gm. carrots.
" 27	360	1.012	115	0	9.27	
Average			**116**			
Feb. 28	65	1.035	114	0	6.70	
" 29	80	1.040	118	18	4.70	
Mar. 1	81	1.040	100	53	4.70	Grain diet: 50 gm. oats. 50 " cracked corn.
" 2	73	1.025	99	54	4.70	
" 3	84	1.019	113	33	4.70	
Average			**108**			
Mar. 4	150	1.015	111	0	6.0	Carrot diet: 500 gm. carrots.
" 5	410	1.015	129	0	9.27	
Average			**120**			

TABLE V.

The Relation of the Character of the Food Intake to P_H of the Urine and to the Elimination of Creatine.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Feb. 16	400	1.015	94	0	9.27	Male rabbit of 2,400 gm. Previous diet unknown.
" 17	450	1.015	144	0	9.27	
" 18	350	1.018	88	0	9.27	Carrot diet: 500 gm. carrots.
Average			**108**			
Feb. 19	400	1.015	110	0	9.27	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 20	100	1.026	84	0	9.27	
" 21	150	1.025	96	0	9.27	
Average			**97**			
Feb. 22	330	1.013	102	33	7.38	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 23	255	1.013	98	50	5.70	
" 24	250	1.012	100	30	5.30	
Average			**100**			
Feb. 25	350	1.012	112	14	9.27	
" 26	450	1.015	122	0	9.27	Carrot diet: 500 gm. carrots.
" 27	390	1.013	86	0	9.27	
Average			**107**			
Feb. 28	90	1.025	121	0	6.90	
" 29	60	1.035	81	7	4.70	
Mar. 1	60	1.025	88	37	4.70	Grain diet: 50 gm. oats. 50 " cracked corn.
" 2	90	1.023	91	64	4.70	
" 3	68	1.025	92	35	4.70	
Average			**95**			
Mar. 4	340	1.010	107	14	9.27	Carrot diet: 500 gm. carrots.
" 5	400	1.016	108	0	9.27	
Average			**107**			

TABLE VI.

The Relation of the Character of the Food Intake to P_H of the Urine and to the Elimination of Creatine.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Feb. 16	300	1.016	100	0	9.27	Male rabbit of 2,000 gm. Previous diet unknown.
" 17	400	1.015	108	0	9.27	
" 18	325	1.015	89	0	9.27	Carrot diet: 500 gm. carrots.
Average			**99**			
Feb. 19	325	1.020	80	0	9.27	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 20	130	1.028	98	0	9.27	
" 21	130	1.020	83	0	9.27	
Average			**87**			
Feb. 22	130	1.020	85	0	7.48	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 23	275	1.010	77	15	5.70	
" 24	180	1.017	76	31	5.30	
Average			**80**			
Feb. 25	275	1.015	91	6	9.27	
" 26	425	1.015	92	0	9.27	Carrot diet: 500 gm. carrots.
" 27	320	1.012	96	0	9.27	
Average			**93**			
Feb. 28	80	1.025	91	0	7.38	Grain diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 29	65	1.030	81	34	5.30	
Mar. 1	45	1.040	90	46	5.30	
" 2	70	1.026	90	42	5.30	
" 3	60	1.020	65	50	5.30	
Average			**83**			
Mar. 4	180	1.015	70	23	9.27	Carrot diet: 500 gm. carrots.
" 5	340	1.018	82	0	9.27	
Average			**76**			

SUMMARY.

Upon a diet of oats and corn, containing an adequate supply of carbohydrate, creatine promptly appears in the urine of the rabbit. A marked condition of acidosis, as measured by the hydrogen ion concentration of the urine is always associated with this phenomenon. Oats and corn are pronounced acid-producing foods. On the other hand, if a base-producing food, such as carrots, is fed to rabbits with creatinuria, this symptom rapidly disappears as the urine becomes alkaline.

The protein *per se* is without special significance in the phenomenon under discussion; for upon a diet consisting of oats, corn, and carrots creatine fails to appear in the urine, and the reaction of the latter remains alkaline. Equally significant is the further fact that the ingestion of hydrochloric acid with the mixed diet causes the appearance in the urine of significant quantities of creatine. Simultaneously the hydrogen ion concentration of the urine is markedly increased.

The conclusion seems justified that there is an interrelationship between acidosis and creatine elimination. Creatine in the urine may prove to be an index of a condition of acidosis in the organism.

STUDIES IN CREATINE METABOLISM.

II. THE INFLUENCE OF ALKALI UPON CREATINE ELIMINATION DURING INANITION.

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(Received for publication, July 31, 1916.)

It is well recognized that during inanition creatine appears in the urine of the rabbit in significant quantities and it is likewise well known that in a starving condition the urine becomes markedly acid in reaction. This change in reaction of the urine has been explained by the assumption that the starving herbivorous animal feeds upon its own tissues in an effort to derive sufficient fuel for its needs and under these circumstances is to all intents and purposes with respect to the character of its metabolism a carnivorous animal. During the process of disintegration of tissue thus devoted to calorific needs acids are formed which must be neutralized by fixed alkalies of the organism and this continual demand for alkali results in base depletion eventually—a concomitant of which is the elimination of creatine in the urine.

According to these ideas, then, in starvation in the rabbit there should be a greatly increased hydrogen ion concentration in the urine. That such a condition obtains may be seen from the data in Table I. Here it is shown that creatinuria and a markedly high hydrogen ion concentration occur simultaneously. These results are in agreement with the hypothesis advanced in the preceding paper; namely, that creatine in the urine is associated with a condition of acidosis and may be independent of the carbohydrate supply. On the other hand, during the creatinuria of inanition in the rabbit[1] and in pernicious vomiting of pregnancy[2] carbohydrate introduction into the body will either

[1] Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213.

[2] Underhill, F. P., and Rand, R. F., *Arch. Int. Med.*, 1910, v, 61.

greatly diminish or totally inhibit the excretion of creatine. This may be explained by assuming that there is diminished tissue breakdown after carbohydrate introduction, hence decreased acid formation, so that base depletion is inhibited more or less completely.

If these ideas are correct, and in particular the view that base depletion is responsible for creatine elimination, it would appear reasonable to assume that during creatinuria, as in inanition, administration of base should either markedly decrease or entirely prevent the appearance of creatine in the urine. The results of four such experiments may be found in Tables II, III, IV and V.

Inspection of these data will show that the subcutaneous injection of 50 cc. of a 2 per cent solution of sodium hydroxide caused the creatine in the urine of three (Tables II, III, and IV) starving rabbits to completely disappear and in a fourth animal (Table V) creatinuria was notably diminished. In only one instance, Table III, did the urine of the injection day, Jan. 16, become alkaline. A similar injection of alkali on the following day was without influence upon creatine excretion in three out of the four experiments. In the fourth, Table V, creatine excretion continued low. A subsequent (Jan. 19) larger administration of base was without significant influence in two instances, Tables II and V; in one, Table III, there was a noticeable drop in creatine excretion and in Table IV it may be observed that creatinuria was completely abolished. In all cases after the final injections the urines became noticeably alkaline.

The experiments clearly demonstrate that administration of alkali may decrease or entirely inhibit the appearance of creatine in the urine during inanition. This effect cannot be regarded merely as a failure of creatine excretion as, for example, by an action upon kidney function, for under these circumstances it might fairly be assumed that creatinine elimination would also be influenced. The data show that creatinine excretion is not changed materially by administration of alkali. The conclusion is therefore justified that alkali checks or inhibits temporarily the formation of creatine, or, viewed from another standpoint, facilitates its further transformation in the organism. The influence of alkali upon creatine elimination is only temporary, as might be expected, inasmuch as the introduction of alkali merely

neutralizes acid produced and does not completely or permanently inhibit acid production. In other words, even though alkali is furnished, acid continues to be formed as the result of the organism's need for fuel. Consequently as the transformation of tissue, in the absence of sufficient glycogen and fat, into energy-producing substances becomes greater and greater the influence of small injections of alkali becomes correspondingly diminished as may be seen from Tables II, III, and V. There seems to be a stage in metabolism where creatine elimination during starvation may be inhibited by administration of alkali. Beyond this point alkali introduction may or may not exert an influence.

TABLE I.

The Influence of Starvation upon the PH *of the Urine and upon Creatine Elimination.*

Date.	Urine.				
	Volume.	Total N.	Creatinine.	Creatine.	PH.
			Rabbit 3.		
1916	*cc.*	*gm.*	*mg.*	*mg.*	
May 1	30	0.56	68	0	7.6
" 2	36	1.04	76	17	5.4
" 3	83	1.71	76	70	4.9
" 4	190	2.58	82	108	4.7
" 5	45	0.34	32	78	5.3
			Rabbit 4.		
May 1	54	0.56	78	0	7.6
" 2	42	0.66	86	20	6.2
" 3	36	0.99	79	55	5.8
" 4	34	0.85	75	95	5.8
" 5	31	1.21	80	90	4.7

TABLE II.

The Influence of Alkali upon Creatine Elimination During Inanition.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Reaction to litmus.	Creatinine.	Creatine.	
1916	*cc.*			*mg.*	*mg.*	Male rabbit of 2,100 gm.
Jan. 12	60	1.035	Acid.	114	0	Fasting.
" 13	60	1.040	"	90	29	"
" 14	50	1.030	"	101	16	"
" 15	40	1.045	"	72	34	"
" 16	50	1.045	"	101	**0**	"
" 17	60	1.030	"	85	**46**	" 10.45 a.m. subcutaneous injection of 50 cc. 2.0 per cent NaOH.
" 18	50	1.035	Alkaline.	90	42	"
" 19	80	1.030	"	95	**60**	"
" 20	60	1.030	"	95	**52**	" 10.45 a.m. and 5.00 p.m. intravenous injection of 50 cc. 2.0 per cent Na_2CO_3.

TABLE III.

The Influence of Alkali upon Creatine Elimination During Inanition.

Date.	Urine.					Remarks.
	Volume.	Specific gravity	Reaction to litmus.	Creatinine.	Creatine.	
1916	*cc.*			*mg.*	*mg.*	Female rabbit of 2,400 gm.
Jan. 12	250	1.010	Acid.	88	13	Fasting.
" 13	45	1.035	"	95	14	"
" 14	50	1.032	"	106	35	"
" 15	50	1.035	"	100	35	"
" 16	50	1.035	Alkaline.	101	**0**	"
" 17	50	1.035	"	80	**93**	" 10.45 a.m. subcutaneous injection of 50 cc. 2.0 per cent NaOH.
" 18	50	1.040	"	101	71	"
" 19	80	1.035	"	90	**27**	"
" 20	40	1.030	"	83	**29**	" 10.45 a.m. and 5.00 p.m. intravenous injection of 50 cc 2.0 per cent Na_2CO_3.

TABLE IV.

The Influence of Alkali upon Creatine Elimination During Inanition.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Reaction to litmus.	Creatinine.	Creatine.	
1916	*cc.*			*mg.*	*mg.*	Female rabbit of 2,100 gm.
Jan. 12	270	1.008	Acid.	70	8	Fasting.
" 13	70	1.040	"	81	10	"
" 14	40	1.035	"	75	18	"
" 15	40	1.035	"	75	17	"
" 16	40	1.025	"	59	**0**	"
" 17	50	1.035	Alkaline.	75	**23**	" 10.45 a.m. subcutaneous injection of 50 cc. 2.0 per cent NaOH.
" 18	40	1.030	"	76	37	"
" 19	90	1.030	"	70	**23**	"
" 20	30	1.035	"	57	**0**	" 10.45 a.m. and 5.00 p.m. intravenous injection of 50 cc. 2.0 per cent Na_2CO_3.

TABLE V.

The Influence of Alkali upon Creatine Elimination During Inanition.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Reaction to litmus.	Creatinine.	Creatine.	
1916	*cc.*			*mg.*	*mg.*	Female rabbit of 2,400 gm.
Jan. 12	170	1.023	Acid.	95	0	Fasting.
" 13	60	1.040	"	101	71	"
" 14	50	1.030	"	85	45	"
" 15	40	1.050	"	90	39	"
" 16	50	1.040	"	95	**7**	"
" 17	80	1.030	Alkaline.	115	**10**	" 10.45 a.m. subcutaneous injection of 50 cc. 2.0 per cent NaOH.
" 18	60	1.035	Acid.	124	65	"
" 19	110	1.030	Alkaline.	89	**68**	"
" 20	70	1.035	"	108	**63**	" 10.45 a.m. and 5.00 p.m. intravenous injection of 50 cc. 2.0 per cent Na_2CO_3.

SUMMARY.

Administration of alkali during the earlier days of starvation may greatly diminish or completely abolish the creatinuria of that condition. Later in the period of inanition introduction of alkali may not show as marked an influence upon the existing creatinuria.

The results of these experiments lend support to the hypothesis that there exists a relationship between acidosis and creatine elimination.

STUDIES IN CREATINE METABOLISM.

III. THE INFLUENCE OF ALKALI UPON THE CREATINURIA OF PHLORHIZIN GLYCOSURIA.

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Creatinuria occurs during phlorhizin glycosuria. Coincident with this there is evidence of carbohydrate deficiency and a well marked acidosis. In view of the fact that in starvation control of the acidosis may lead to disappearance of creatine from the urine of the rabbit it was deemed of importance to determine what influence is to be exerted upon the creatinuria provoked by phlorhizin upon administration of relatively large quantities of sodium bicarbonate.

Methods.

Throughout the period of investigation the dogs employed were allowed to fast. The animals were given subcutaneous doses of 1 gm. of phlorhizin dissolved in 10 cc. of olive oil on 2 successive days previous to the experimental period and thereafter 1 gm. every alternate day. Each day was divided into two 6 hour periods and one 12 hour period, by catheterization. For the estimation of sugar the method of Benedict[1] was employed and creatine was determined according to the procedure of Graham and Poulton.[2] Sodium bicarbonate was administered by mouth in gelatin capsules during the 6 hour periods but was not given during the 12 hour periods.

It is apparent from the data presented in Tables I and II that *the administration of relatively large quantities of sodium bicar-*

[1] Benedict, S. R., *J. Biol. Chem.*, 1911, ix, 57.

[2] Graham, G., and Poulton, E. P., *Proc. Roy. Soc., Series B*, 1914, lxxxvii, 205.

bonate is without appreciable influence upon the elimination of creatine during phlorhizin glycosuria. This is equally true whether the animal is in a state of complete phlorhizination or not. The preceding paper has shown that administration of alkali is much more potent in abolishing creatinuria in the early days of starvation than it is in the later periods of inanition, perhaps because of the greater difficulty of controlling the condition of acidosis. In the present work it is possible that the same reasoning may hold also, that is, that during phlorhizin intoxication the occasion for acid production is so great that its successful control is improbable. In line with this view stands the fact that the ingestion of alkali as carried through in these experiments seemed to have little or no influence upon the excretion of ketone substances judged by qualitative tests. On the other hand, it would seem reasonable to assume that the giving of these large quantities of alkali should exert at least a slight action upon creatine excretion if the condition of acidosis, that is, alkali depletion, is to be considered as the stimulus for the production of creatine. One is therefore forced to the conclusion that *more than one factor may govern the mechanism leading to creatinuria.*

TABLE I.

The Influence of Alkali upon Creatine Excretion in Phlorhizin Glycosuria.

Date.	Urine.										Remarks.
	Total N.		Dextrose.		Creatinine N.		Creatine N.		D: N.		
	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	
1916	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*			
June 6	1.99	4.85	20.82	34.55	45	100	85	19	10.25	7.12	June 4 and 5 each, 1 gm. phlorhizin.
" 7	1.18		19.83		46		78		16.80		1 gm. phlorhizin.
	1.73	2.91	14.51	34.34	57	103	21	99	8.40	11.78	4 " $NaHCO_3$. per hr.
		5.46		21.24		63		110		3.90	No $NaHCO_3$.
" 8	1.88		14.22		39		50		7.56		2 gm. $NaHCO_3$
	1.96	3.84	13.60	27.82	49	88	45	95	6.95	7.24	per hr.
		3.02		6.80		59		100		2.25	No $NaHCO_3$.
" 9	2.80		9.80		34		54		3.50		1 gm. phlorhizin.
	1.51	4.31	20.50	30.30	31	65	61	105	13.60	7.02	2 gm. $NaHCO_3$. per hr.
		2.07		7.90		42		129		3.82	No $NaHCO_3$.

Urines obtained during periods of alkali administration were strongly alkaline. During the other periods urines were acid.

TABLE II.

The Influence of Alkali upon Creatine Excretion in Phlorhizin Glycosuria.

Date.	Urine.										Remarks.
	Total N.		Dextrose.		Creatinine N.		Creatine N.		D: N.		
	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	
1916	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg*	*mg.*	*mg.*			
May 29	1.32		5.98		22		36		4.52		May 27 and 28, 1 gm. phlorhizin.
	1.43	2.75	5.55	11.53	20	42	40	76	3.85	4.20	May 29, no $NaHCO_3$.
" 30		2.27		7.87		26		90		3.47	No Na_2CO_3.
	1.60		6.12		17		49		3.82		1 gm. phlorhizin.
	1.57	3.17	5.68	11.80	15	32	50	99	3.62	3.72	1 " $NaHCO_3$ per hr.
" 31		2.20		10.30		29		102		4.70	No $NaHCO_3$.
	0.98		3.56		12		51		3.62		2 gm. $NaHCO_3$
	1.07	2.05	3.41	6.97	12	24	54	105	3.18	3.40	per hr.
June 1		1.48		6.62		22		83		4.48	No Na_2CO_3.
	0.92		2.64		10		36		2.86		1 gm. phlorhizin.
	0.67	1.59	2.00	4.64	8	18	27	63	2.98	2.92	2 " $NaHCO_3$ per hr.

Urines obtained during periods of alkali administration were alkaline. During the other periods urines were acid.

STUDIES IN CREATINE METABOLISM.

IV. THE RELATIONSHIP OF CREATINURIA TO CARBOHYDRATE METABOLISM AND ACIDOSIS.

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In 1908 it was shown by Underhill and Kleiner[1] that the subcutaneous injection of hydrazine sulfate called forth, among other symptoms, a condition of marked creatinuria. The creatine eliminated under these circumstances was much greater than could be accounted for by the attendant starvation. Later work[2] demonstrated that hydrazine injections resulted invariably, in dogs, and occasionally in rabbits, in a state of distinct hypoglycemia without the appearance in the urine of any reducing substance and with a temporary disappearance of glycogen from the liver and muscles, accompanied by an increased respiratory quotient.[3] Recently MacAdam[4] has called attention to the fact that hypoglycemia may be produced invariably in rabbits provided the dose of hydrazine is sufficiently large. The same investigator has also pointed out that there is a close association between the condition of hypoglycemia and creatinuria for "after hydrazine injection in rabbits (a) if no alteration in the sugar content of the blood occurs, no marked creatinuria results; (b) but the excretion of creatine is associated with a condition of hypoglycemia." From these results it would appear that support is afforded the current view concerning creatinuria; namely, that creatine in the urine is closely associated with a deficiency of carbohydrate in the body.

[1] Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 165.

[2] Underhill, F. P., *J. Biol. Chem.*, 1911, x, 159; also Underhill, F. P., and Hogan, A. G., *ibid.*, 1915, xx, 203.

[3] Underhill, F. P., and Murlin, J. R., *J. Biol. Chem.*, 1915, xxii, 499.

[4] MacAdam, W., *Biochem. J.*, 1915, ix, 229.

As outlined in the preceding papers[5] creatine may find its way into the urine of the rabbit at least under circumstances which exclude the possibility of carbohydrate deficiency. On the other hand, the data presented in the papers to which reference is made indicate that creatinuria is connected with a condition of acidosis in the sense of withdrawal of base from the body. Acidosis and carbohydrate deficiency usually accompany one another, indeed the first may many times be regarded as the sequel of the latter. In hydrazine one has an excellent example of carbohydrate deficiency. The question therefore naturally arises, whether the creatinuria induced by hydrazine is caused by carbohydrate depletion or by an accompanying acidosis. Or must it be recognized that creatine in the urine may arise as a result of two entirely different types of mechanisms being set in motion? May creatine appear in the urine with deficiency of carbohydrate without acidosis and may creatinuria be present in conditions of acidosis with a sufficiency of carbohydrate in the body? The latter part of the question has already been answered in the affirmative by the data in the preceding papers.[5] The query whether creatine may be excreted in the urine under circumstances where carbohydrate depletion prevails without an accompanying acidosis demands an answer which it is the object of the present communication to furnish and for this purpose the creatinuria induced by hydrazine has been studied in the dog.

Methods.

Inasmuch as animals refuse to eat after hydrazine administration it becomes essential to determine the influence of starvation upon the factors under discussion before proceeding to a consideration of the effect of hydrazine. Accordingly dogs were allowed to starve for a period of 5 days, urine being collected in 24 hour periods by catheterization, precautions being used to prevent bladder infection. With these specimens of urine determination was made of the total nitrogen, the creatinine[6] nitrogen, and the creatine[7] nitrogen. The sugar content of the blood drawn from the ear vein was estimated by the method of

[5] Underhill, *J. Biol. Chem.*, 1916, xxvi, 127, 141, 147.

[6] Folin's method.

[7] Benedict and Myers' method.

Benedict and Lewis.[8] Following the period of inanition the dogs were well fed on a mixed diet over a period sufficient to allow them to regain their normal body weight. At this time they were given subcutaneous injections of hydrazine sulfate in the dosage of 50 mg. per kilo body weight and estimations were made upon the urine and blood as detailed above, except that 12 hour periods were employed instead of 24 hour periods. This was done especially for the purpose of correlating more closely changes in blood sugar and creatine of the urine. Where determinations of the hydrogen ion concentration of the urine were carried through the procedure of Henderson and Palmer[9] was employed.

The data in Tables I, II, III, and IV show very clearly that hydrazine induces a marked creatinuria which is far in excess of that provoked by starvation. The appearance of creatine in the urine in significant amounts is coincident with the period of hypoglycemia. With the return of blood sugar to normal creatinuria falls in general, in one instance to the starvation level. It is evident then that creatinuria and hypoglycemia are closely associated and it is probable that the appearance in the urine of creatine is dependent upon the hypoglycemia. It is also to be noted that the total nitrogen of the urine during the hydrazine period is markedly higher than with simple inanition. From this one might query whether the creatine was derived from tissue disintegration induced by the condition of hypoglycemia.

Hypoglycemia after hydrazine intoxication is not associated with the appearance in the urine of any of the typical compounds usually connected with the term acidosis, as ketone substances, lactic acid, etc. This has been demonstrated by Underhill and Kleiner[1] and by MacAdam.[4] It became of interest therefore to determine the hydrogen ion concentration of the urine as an index to the changes taking place in the body with regard to the acid-base equilibrium. In the dog this is exceedingly difficult of accomplishment where catheterization is practiced, owing to the fact that error is likely to be introduced in the interpretation of the results. For example, if the urine showed a change from an acid to an alkaline reaction it would be difficult to determine convincingly whether the change had occurred in the

[8] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[9] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393.

bladder or elsewhere. Again, in order to insure freedom from bladder infection it is customary in this laboratory to allow a few cc. of boric acid to remain in the bladder after catheterization. It became necessary, therefore, to determine the hydrogen ion concentration upon samples which had been voided spontaneously. The results of such a study first during a preliminary starvation period and then after hydrazine administration are given in Table V. In general, during the period of inanition the urine became progressively more acid *whereas in the hydrazine period the distinctly acid urine voided at the beginning of the period showed a marked temporary change to alkalinity* followed by a resumption of the acidity. The decrease in hydrogen ion concentration corresponds well with the period of low blood sugar as determined by previous investigations since hypoglycemia obtains first from the 36th to the 48th hour after administration of hydrazine and persists for less than 48 hours. The alkalinity of the urine was undoubtedly due to the presence of a carbonate since on acidification carbon dioxide was given off.

These results make it evident that in hydrazine intoxication the increased creatinuria cannot be ascribed to a condition of marked acidosis. Taken in connection with the data already reported they also lead to the conclusion that in the consideration of the cause of creatinuria at least two types of factors may be potent. In the first place creatine may appear in the urine in conditions of acidosis where the question of carbohydrate deficiency is not involved, and secondly, a marked creatinuria may be induced during carbohydrate deficiency even when acidosis is absent. A glance at Tables I to IV will reveal one other characteristic effect of hydrazine; namely, a noticeably increased output of nitrogen when comparison is made with corresponding figures for the period of simple starvation. Will this increased nitrogen output indicating tissue disintegration account for the augmented creatine excretion?

The observations here presented repeat once again the relationship existing between a state of alkalosis and blood sugar content. A previous communication[10] has demonstrated that introduction of alkali into rabbits may at times appreciably lower blood sugar content. After thyreoparathyroidectomy[11] in dogs

[10] Underhill, *J. Biol. Chem.*, 1916, xxv, 463.

[11] Underhill, F. P. and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87.

blood sugar may fall markedly and at this period a condition of alkalosis[12] exists. With hydrazine hypoglycemia it is probable that the same state is present. It is apparent therefore that *there is a close relationship between blood sugar content and the acid-base equilibrium in the body.*

TABLE I.

The Influence of Hydrazine upon the Excretion of Creatine.

Date.	Body weight.	Urine. Total N. 12 hr. period.	Total N. 24 hr. period.	Creatinine N. 12 hr. period.	Creatinine N. 24 hr. period.	Creatine N. 12 hr. period.	Creatine N. 24 hr. period.	Creatinine N. 12 hr. period.	Creatinine N. 24 hr. period.	Creatine N. 12 hr. period.	Creatine N. 24 hr. period.	Blood sugar.	Remarks.
Starvation period.													
1916	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
May 2	7.5		4.29		43		3		1.0		0.07	0.096	Bitch of 7.5 kilos. Last meal 24 hrs. previous to beginning of experiment.
" 3	7.2		1.96		44		3		2.0		0.15	0.096	
" 4	7.2		2.40		36		4		1.5		0.17	0.103	
" 5	7.1		2.22		44		3		2.0		0.14	0.102	
" 6	6.9		2.22		43		4		2.0		0.18	0.094	
Hydrazine period.													
May 15	7.7	0.53		8		12		1.5		2.3		0.107	Animal well fed on mixed diet from May 7 to 14.
		1.42	1.95	20	28	14	26	1.4	1.4	1.0	1.3	0.123	
" 16	7.2	1.40		20		23		1.4		1.6		0.113	May 15, 9 a.m., subcutaneous injection of hydrazine sulfate, 50 mg. per kilo. No food after injection.
		1.98	3.38	25	45	39	62	1.3	1.3	2.0	1.8	0.135	
" 17	7.1	2.05		28		33		1.4		1.6		0.078	
		1.88	3.93	29	57	35	68	1.5	1.4	1.9	1.7	0.109	
" 18	7.0	2.01		23		35		1.2		1.7		0.107	
		1.06	3.07	20	43	38	73	1.9	1.4	3.6	2.4		
" 19	6.8	0.99		18		20		1.8		2.0			
		0.85	1.84	19	37	11	31	2.2	2.0	1.3	1.7	0.100	

[12] Wilson, D. W., Stearns, T., and Thurlow, M. D., *J. Biol. Chem.*, 1915, xxiii, 100. Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *ibid.*, 1915, xviii, 123.

TABLE II.

The Influence of Hydrazine upon the Excretion of Creatine.

Date.	Body weight.	Urine. Total N. 12 hr. period.	Total N. 24 hr. period.	Creatinine N. 12 hr. period.	Creatinine N. 24 hr. period.	Creatine N. 12 hr. period.	Creatine N. 24 hr. period.	Creatinine N. 12 hr. period.	Creatinine N. 24 hr. period.	Creatine N. 12 hr. period.	Creatine N. 24 hr. period.	Blood sugar.	Remarks.
Starvation period.													
1915	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Nov. 8	16.0		5.22		118		16		2.2		0.3	0.15	Bitch of 16 kilos. Last meal 24 hrs. previous to beginning of experiment.
" 9	15.1		3.82		196		7		5.1		0.2	0.14	
" 10	14.8		2.98		205		5		6.8		0.1	0.13	
" 11	14.6		2.48		174		10		7.0		0.4	0.14	
" 12	14.4		2.25		154		37		6.8		1.6	0.11	
Hydrazine period.													
Dec. 6	15.1	2.28		78		8		3.4		0.3		0.15	Animal well fed on mixed diet from Nov. 13 to Dec. 5. Dec. 6, 9 a.m., subcutaneous injection of hydrazine sulfate, 50 mg. per kilo.
		3.14	5.42	80	158	17	25	2.5	2.9	0.5	0.4	0.12	
" 7	14.2	3.47		89		78		2.5		2.2		0.06	
		4.23	7.70	102	191	98	176	2.4	2.4	2.3	2.2	0.04	
" 8	14.1	4.65		87		49		1.9		1.0		0.11	
		5.16	9.91	77	164	61	110	1.4	1.6	1.1	1.1	0.16	
" 9	13.9	4.70		86		94		1.9		2.0		0.11	
		3.66	8.36	64	150	122	216	1.7	1.8	3.3	2.5	0.12	
" 10	13.5	4.55		155		86		3.4		1.9		0.12	

TABLE III.

The Influence of Hydrazine upon the Excretion of Creatine.

Date.	Body weight.	Urine. Total N. 12 hr. period.	Total N. 24 hr. period.	Creatinine N. 12 hr. period.	Creatinine N. 24 hr. period.	Creatine N. 12 hr. period.	Creatine N. 24 hr. period.	Creatinine N. 12 hr. period.	Creatinine N. 24 hr. period.	Creatine N. 12 hr. period.	Creatine N. 24 hr. period.	Blood sugar.	Remarks.
Starvation period.													
1915	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Nov. 16	6.2		2.10		56		0		2.6		0.1	0.11	Bitch of 6.2 kilos.
" 17	6.1		1.53		50		7		3.2		0.4	0.12	Last meal 24
" 18	6.0		1.40		61		20		4.3		0.1	0.13	hrs. previous
" 19	5.9		1.34		56		7		4.1		0.05	0.12	to beginning of
" 20	5.7		1.39		58		17		4.1		0.1	0.11	experiment.
Hydrazine period.													
1916													
Jan. 31	6.3	1.33		25		0		1.8		0		0.12	Animal well fed
		1.89	3.22	24	49	9	9	1.2	1.5	0.4	0.2	0.13	on mixed diet
Feb. 1	5.8	1.67		22		14		1.3		0.8		0.12	from Nov. 21
		2.26	3.93	25	47	27	41	1.1	1.2	1.2	1.0	0.08	until Jan. 30.
" 2	5.7	2.00		25		24		1.2		1.1		0.03	Jan. 31, 9 a.m.
		2.10	4.10	27	52	22	46	1.2	1.2	1.0	1.1	0.04	subcutaneous
" 3	5.7	1.68		26		28		1.5		1.7		0.13	injection of
		1.44	3.12	24	50	9	37	1.6	1.6	0.6	1.2	0.12	hydrazine sul-
" 4	5.6	1.28		23		3		1.8		0.2		0.12	fate, 50 mg. per
		1.15	2.43	26	49	1	4	2.2	2.0	0.1	0.1	0.12	kilo.
" 5	5.5	1.05		25	49	0.6		2.3		0.05			

TABLE IV.

The Influence of Hydrazine upon the Excretion of Creatine.

Date.	Body weight.	Urine. Total N. 12 hr. period.	Total N. 24 hr. period.	Creatinine N. 12 hr. period.	Creatinine N. 24 hr. period.	Creatine N. 12 hr. period.	Creatine N. 24 hr. period.	Creatinine N. 12 hr. period.	Creatinine N. 24 hr. period.	Creatine N. 12 hr. period.	Creatine N. 24 hr. period.	Blood sugar.	Remarks
Starvation period.													
1916	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
May 2	6.4		2.16		40		0		1.9		0	0.087	Bitch of 6.4
" 3	6.2		2.88		44		2		1.5		0.07	0.088	kilos. Last
" 4	6.0		1.82		50		12		2.7		0.66	0.094	meal 24 hrs.
" 5	5.9		1.77		53		17		3.0		0.96	0.092	previous to
" 6	5.8		1.90		53		15		2.8		0.80	0.089	beginning of experiment.
Hydrazine period.													
May 15	6.6	1.09		18		3		1.6		0.28		0.11	Animal well fed
		1.49	2.58	20	38	11	14	1.3	1.5	0.74	0.5		on mixed diet
" 16	6.1	1.10		18		12		1.6		1.1		0.126	from May 7 to
		1.20	2.30	18	36	42	54	1.5	1.6	3.5	2.3	0.140	14. Last meal
" 17	5.9	1.51		23		37		1.5		2.4		0.079	on May 14.
		1.55	3.06	24	47	36	73	1.5	1.5	2.3	2.4	0.102	May 15, 9 a.m.,
" 18	5.8	1.45		18		28		1.2		1.9		0.099	subcutaneous
		0.98	2.43	16	34	35	63	1.6	1.4	3.6	2.6		injection of
" 19	5.8	0.89		24		29		2.7		3.3		0.098	hydrazine sul-
		0.84	1.73	20	44	4	33	2.4	2.5	0.48	1.9		fate, 50 mg.
" 20	5.8	0.83		18		12		2.2		1.4		0.098	per kilo. No food after injection.

TABLE V.

The Influence of Starvation and of Hydrazine upon the Hydrogen Ion Concentration of the Urine.

Date.	Dog 9.		Dog 10.	
	Time of micturition.	P_H.	Time of micturition.	P_H.
Starvation period. Last meal May 8, 10 a.m.				
May 8	6 p.m.	7.14		
" 9	9 a.m.	7.00	3 p.m.	7.40
" 10	9 "	7.38		
	5 p.m.	7.00		
" 11	9 a.m.	6.90	9 a.m.	7.00
	5 p.m.	6.90		
" 12	9 a.m.	6.30		
" 13			9 "	6.00
Hydrazine period. May 25, subcutaneous injection of 50 mg. per kilo hydrazine sulfate.				
May 26	9 a.m.	5.70	9 a.m.	6.15
" 27	10 "	5.70	12 m.	6.30
	5 p.m.	6.70	5 p.m.	6.30
" 28	9 a.m.	7.41	10 a.m.	9.27
	3 p.m.	7.45		
	9 "	6.70		
" 29	9 a.m.	5.50	9 a.m.	7.38
			9 p.m.	6.00
" 30			9 a.m.	5.70

SUMMARY.

The subcutaneous administration of hydrazine to dogs induces a marked creatinuria which in general closely parallels the period of hypoglycemia provoked. These observations corroborate the results of previous investigations.

During the period of hypoglycemia and most marked creatinuria the hydrogen ion concentration of the urine is greatly depressed—even to the point of marked alkalinity. The alkalinity is probably due to the presence of carbonates.

The relationship of hypoglycemia and alkalosis again emphasizes the significance of acid-base equilibrium in the regulation of the blood sugar content.

The results here presented make it evident that creatine elimination in the urine may be induced by at least two different sets of conditions (1) creatine may appear in the urine in states of acidosis where carbohydrate deficiency is not involved and (2) creatinuria may be present during carbohydrate deficiency even in the absence of acidosis.

ALTERATIONS IN THE OUTPUT OF CERTAIN URINARY CONSTITUENTS AS DETERMINED BY CHANGES IN THE CHARACTER OF THE DIET.

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Changes in the character of the diet may induce the appearance of creatine in the urine or may cause it to disappear therefrom. Rabbits fed upon a mixture of corn and oats excrete creatine in the urine. A carrot diet causes the urinary creatine to disappear. The character of the protein of the diet plays little or no rôle since the urine eliminated by rabbits upon a mixture of corn, oats, and carrots remains creatine-free. By the addition of hydrochloric acid to the mixed diet creatinuria is induced. Since corn and oats are acid-producing foods and carrots are base producers, the facts noted above with respect to the appearance or abolishment of creatinuria have been explained by the hypothesis that a condition of acidosis, in the sense of base withdrawal from the body, is responsible for the appearance of creatine in the urine. This condition may be abolished or prevented by the ingestion of base-producing foods, like carrots.

In view of these facts it seemed desirable to determine whether changing the character of the diet in the manner indicated above would effect alterations in the output of other urinary constituents, consideration of course being given to the differences in intake of various components of the diet. This attempt forms the subject of the present paper, the results obtained being detailed in the tables appended.

Methods.

The urine was divided into 24 hour periods by expressing it from the bladder by pressure through the abdominal wall. Total nitrogen in the urine was estimated by the Kjeldahl method; creatinine by the Folin method; creatine according to Benedict and Myers; hydrogen ion concentration by the procedure of Henderson and Palmer; ammonia by the Folin aeration method; phosphorus by titration with uranium nitrate; and calcium according to McCrudden. Phosphorus in the food was estimated by the Neumann method. The "grain diet" consisted of 50 gm. cracked corn and 50 gm. oats. The "carrot diet" contained 500 gm. fresh carrots. The "mixed diet" consisted of 25 gm. cracked corn, 25 gm. oats, and 250 gm. carrots. During the acid period, hydrochloric acid was administered by stomach tube twice daily at intervals of 8 hours in the dosage of 50 cc. 0.25 N HCl. The variations in the intake of nitrogen and phosphorus on the grain diet are due to the variable amounts of food actually ingested.

The one striking feature of the experiments may be seen by inspection of the tables relative to the grain diet. Compared with the intake *the urinary phosphorus output* is very large. During no other period did this condition obtain. Inasmuch as at this interval the hydrogen ion concentration of the urine was greatest it is probable that the excessive elimination of phosphorus in this instance at least may be accepted as a means through which the rabbit regulates the acid-base equilibrium of the organism. Ammonia plays little rôle here apparently for in no case was there a significant excretion of this constituent during the grain period. On the other hand, the elimination of ammonia in the rabbit is very difficult of interpretation for although the ammonia output in Rabbits 2 and 3 was negligible throughout irrespective of the character of the diet significant quantities of ammonia were excreted through the kidney by Rabbit 1, even though a base-producing diet was fed. The output was greatest when hydrochloric acid was administered although not much greater than that observed without added acid. With Rabbit 4 ammonia excretion was markedly increased only during the period of hydrochloric acid feeding. It is probable,

then, that ammonia may play a part in the acid-base equilibrium of the body under certain conditions but the exact circumstances which allow or prevent this type of regulation in this manner remain to be determined.

Urinary calcium output seems to be so variable under the experimental conditions that no conclusion regarding the significance of this element seems justified.

There is the same parallelism between creatine output and hydrogen ion concentration of the urine observed in previous papers from this laboratory upon the subject.

The experiments demonstrate beyond doubt that the excretion of creatine during the grain diet cannot be explained by insufficiency of food intake since in all instances the animals were undoubtedly in nitrogen equilibrium as indicated by the probable positive nitrogen balance. During the carrot diet, on the other hand, a negative nitrogen balance is always in order; but creatinuria does not develop, hence it is evident that the creatinuria observed upon the grain diet cannot be explained by food insufficiency.

The tables follow.

TABLE I.

Rabbit 1, Male, 2,360 Gm., Weight April 14, 2,330 Gm.

Date.	Diet.	Intake (food).		Urine.								
		Nitrogen.	Phosphorus.	Volume.	Specific gravity.	Total N.	NH_3 N.	Creatinine.	Creatine.	P_H.	Phosphorus.	Calcium.
1916		*mg.*	*mg.*	*cc.*	*1.0—*	*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*
Mar. 30	Mixed.	1,115	180	145	20	684	4	77	0	9.27	134	15
" 31	Grain.	1,060	84	60	32	920	0	147	27	4.70	201	10
April 1	"	1,060	84	43	43	806	0	124	37	4.90	164	6
" 2	"	1,060	84	40	48	786	0	115	56	4.90	151	6
" 3	"	1,364	108	34	60	830	5	104	38	4.90	145	6
" 4	"	1,288	102	33	55	890	0	97	44	4.90	142	11
" 5	"	954	76	33	50	870	4	81	44	5.30	138	9
Average		1,131	89	41	1.044	850	1	111	41	4.90	157	8
April 6	Carrot.	715	240	160	20	830	5	81	34	9.27	91	8
" 7	"	715	240	385	14	1,102	20	108	0	9.27	93	8
" 8	"	715	240	354	16	1,092	13	90	0	8.00	97	10
Average		715	240	299	1.016	1,008	12	93	11	8.81	93	8
April 9	Mixed.	1,115	180	160	20	699	31	68	0	8.70	64	18
" 10	"	1,115	180	150	21	783	38	85	0	8.70	47	25
Average		1,115	180	155	1.020	741	34	76	0	8.70	55	16
April 11	Mixed +HCl.	1,115	180	238	11	739	49	84	48	8.00	66	18
" 12	"	1,115	180	240	11	727	40	91	51	7.38	80	17
" 13	"	1,115	180	190	30	658	31	95	28	6.90	60	10
Average		1,115	180	222	1.017	702	40	90	42	7.42	68	15
April 14	Carrot.	715	240	300	21	776	18	122	0	9.27	70	7
" 15	"	715	240	390	14	1,176	45	120	0	9.27	127	16
" 16	"	715	240	332	14	1,155	14	102	0	9.27	120	11
Average		715	240	340	1.016	1,035	25	114	0	9.27	105	11

TABLE II.

Rabbit 2, Male, 3,420 Gm., Weight April 14, 3,100 Gm.

Date.	Diet.	Intake (food).		Urine.								
		Nitrogen.	Phosphorus.	Volume.	Specific gravity.	Total N.	NH_3 N.	Creatinine.	Creatine.	P_H.	Phosphorus.	Calcium.
1916		*mg.*	*mg.*	*cc.*	*1.0—*	*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*
Mar. 30	Mixed.	1,115	180	290	17	1,379	0	162	0	9.27	188	32
" 31	Grain.	1,060	84	93	20	699	0	95	0	7.48	155	28
April 1	"	1,060	84	47	20	386	0	70	0	6.00	85	7
" 2	"	1,060	84	190	22	1,675	3	190	21	5.70	303	18
" 3	"	1,060	84	80	17	673	6	97	13	6.00	125	20
" 4	"	1,060	84	142	16	1,159	0	122	53	5.70	213	27
" 5	"	833	66	110	13	913	0	119	50	5.70	163	22
Average		1,022	81	110	1.018	917	1	115	23	6.09	174	20
April 6	Carrot.	715	240	285	10	1,381	15	142	0	8.70	118	27
" 7	"	715	240	394	12	1,142	0	95	0	9.27	76	15
" 8	"	715	240	510	15	1,679	0	146	0	8.00	124	66
Average		715	240	396	1.012	1,400	5	127	0	8.65	106	36
April 9	Mixed.	1,115	180	214	20	1,000	0	106	0	8.70	59	61
" 10	"	1,115	180	156	18	843	0	108	0	8.70	89	49
Average		1,115	180	185	1.019	921	0	107	0	8.70	74	55
Apri 11	Mixed +HCl.	1,115	180	328	12	1,513	0	187	0	8.00	179	31
" 12	"	1,115	180	290	12	1,218	3	130	56	4.90	188	62
" 13	"	1,115	180	174	37	1,166	3	125	59	5.70	168	56
Average		1,115	180	264	1.020	1,299	2	147	38	6.20	178	49

TABLE III.

Rabbit 3, Male, 2,320 Gm., Weight April 11, 2,280 Gm.

Date.	Diet.	Intake (food).		Urine.								
		Nitrogen.	Phosphorus.	Volume.	Specific gravity.	Total N.	NH_3 N.	Creatinine.	Creatine.	P_H.	Phosphorus.	Calcium.
1916		*mg.*	*mg.*	*cc.*	*1.0—*	*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*
Mar. 30	Mixed.	1,115	180	145	20	583	8	85	0	9.27	91	50
" 31	Grain.	1,060	84	60	26	684	0	101	0	8.00	130	39
April 1	"	1,060	84	53	33	769	3	108	8	6.00	138	31
" 2	"	1,060	84	54	34	806	0	101	8	5.70	128	47
" 3	"	909	72	59	30	811	0	113	14	5.70	149	88
" 4	"	909	72	63	23	874	1	84	23	5.70	130	66
" 5	"	682	54	64	27	850	0	81	39	5.70	128	63
Average		946	75	59	1.029	799	0.6	98	15	6.13	133	55
April 6	Carrot.	715	240	174	17	1,037	1	96	0	8.70	72	71
" 7	"	715	240	350	15	1,143	0	108	0	8.70	96	94
" 8	"	715	240	326	17	723	0	100	0	8.00	100	127
Average		715	240	283	1.018	967	0.3	101	0	8.46	89	97
April 9	Mixed.	1,115	180	190	17	729	0	90	0	8.00	117	177
" 10	"	1,115	180	172	17	611	0	101	0	8.00	106	142
Average		1,115	180	180	1.017	820	0	95	0	8.00	111	159

TABLE IV.

Rabbit 4, Male, 1,980 Gm., Weight April 11, 2,060 Gm.

Date.	Diet.	Intake (food).		Urine.								
		Nitrogen.	Phosphorus.	Volume.	Specific gravity.	Total N.	NH_3 N.	Creatinine.	Creatine.	P_H.	Phosphorus.	Calcium.
1916		*mg.*	*mg.*	*cc.*	*1.0—*	*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*
Mar. 30	Mixed.	1,115	180	155	18	546	0	82	0	9.27	81	26
" 31	Grain.	1,060	84	45	35	768	0	90	0	8.70	134	10
April 1	"	1,060	84	28	50	454	0	81	0	6.30	99	14
" 2	"	1,060	84	25	55	543	4	95	6	6.00	109	29
" 3	"	1,060	84	40	47	695	0	73	16	5.30	140	7
" 4	"	757	60	40	47	763	2	61	22	5.70	157	28
" 5	"	757	60	41	43	662	0	62	22	5.70	138	42
Average		959	76	36	1.046	647	1	77	11	6.28	129	21
April 6	Carrot.	715	240	270	12	1,010	5	101	13	9.27	80	23
" 7	"	715	240	402	13	985	3	86	0	8.70	95	24
" 8	"	715	240	380	15	759	0	73	0	8.70	72	19
Average		715	240	350	1.013	918	3	87	4	8.89	82	22
April 9	Mixed.	1,115	180	158	20	736	5	75	0	8.70	76	20
" 10	"	1,115	180	158	19	638	7	81	0	8.70	76	46
Average		1,115	180	158	1.019	687	6	78	0	8.70	76	33
April 11	Mixed	1,115	180	262	12	794	12	109	0	7.48	115	37
" 12	+HCl.	1,115	180	206	21	1,108	25	67	73	5.30	141	48
Average		1,115	180	234	1.016	951	18	88	36	6.39	128	42

SUMMARY.

Upon a diet of corn and oats the output of phosphorus in the urine of rabbits is far in excess of the intake of this element in the food. Since the hydrogen ion concentration of the urine is very high under these circumstances the great excess of phosphoric acid in the urine may be regarded as a method of regulating acid-base equilibrium in the body of the rabbit.

Ammonia excretion is variable under changing conditions of diet, and although it appears that this base may function as a neutralizing agent at times, further experiments are necessary before the laws of ammonia excretion in the rabbit can be formulated.

Calcium elimination is too variable to justify inferences being drawn under the experimental conditions.

The results obtained indicate that the creatinuria observed upon a grain diet cannot be explained upon the hypothesis of food insufficiency.

THE INTERRELATIONS OF BLOOD FAT AND BLOOD SUGAR CONTENT OF DOGS UNDER THE INFLUENCE OF HYDRAZINE.

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The metabolism of carbohydrate and that of fat are closely associated and interdependent. This relationship is more evident under conditions of abnormal metabolic activity than it is when the nutritive rhythm remains undisturbed. The most striking instances of the interrelationships of fat and carbohydrate in metabolism are to be observed in the pathological states induced by intoxication with phosphorus, phlorhizin, and hydrazine, and in alcoholism and diabetes, which are characterized by the so called "fatty" livers.[1] In general a "fatty" liver is associated with a deficiency of carbohydrate in that organ. The "sugar-hungry cells" attract fat to themselves in much greater quantity than they can burn it.[2] This implies transport of fat through the blood stream. Under ordinary nutritive circumstances the fat content of dogs is quite constant for the individual and for the species, which indicates an efficient regulation.[3] The blood fat content therefore may be regarded as one of the body constants like the blood sugar content; and like it is subject to deviation by a variety of agencies. The factors which are potent in causing changes in blood fat content have not been sufficiently studied to correlate them with those which are effective in producing alterations in blood sugar content.

It has been demonstrated repeatedly that administration of

[1] For a discussion of the subject see Rosenfeld, *Ergebn. Physiol.*, 1902, i, 651; 1903, ii, 50.

[2] Mandel, A. R., and Lusk, G., *Am. J. Physiol.*, 1906, xvi, 129.

[3] Bloor, W. R., *J. Biol. Chem.*, 1914, xix, 1.

hydrazine to dogs causes low blood sugar content, loss of glycogen from both the liver and muscles, and induces a "fatty" liver. The "fatty" liver presupposes fat infiltration with a consequent transport through the blood stream. Is the "fatty" liver produced by the storage of fat from the blood, the amount of fat in the latter remaining unchanged, or is there an actual increase of the fat of the blood during hydrazine intoxication? If blood fat is augmented under these circumstances does it bear any relation to blood sugar content? These are questions which seem of fundamental importance in a consideration of the mechanism of blood fat regulation. They form the subject of the present investigation.

Methods.

Dogs under the influence of hydrazine refuse food or fail to retain it. Hence a fasting condition is inevitable in hydrazine experimentation. Results obtained by the action of this poison should therefore be interpreted with respect to the effects produced by fasting alone. Accordingly dogs were fasted for periods of 6 days, determinations of the blood fat and blood sugar being made daily upon samples obtained from a marginal ear vein. Blood fat was estimated by the method of Bloor[4] with a Richards nephelometer. Blood sugar content was determined in part by the procedure of Forschbach and Severin and in part by that of Benedict and Lewis. Subsequent to the fasting period the animals were well fed with mixed diets for varying intervals and were then given subcutaneous injections of hydrazine sulfate in the dosage of 50 mg. per kilo body weight. Blood fat and blood sugar content were then determined as in the fasting period except that the estimations were carried through at 12 hour intervals.

The results obtained with five dogs are presented in the table. During the fasting period there is a general tendency for blood fat content to diminish in the first days of inanition. This decrease is followed by an augmentation of the blood fat somewhat beyond the initial value. The outcome of these experiments is somewhat different from that of Bloor who found either

[4] Bloor, *J. Biol. Chem.*, 1915, xxiii, 317.

Date.	Body weight.	Blood sugar.	Blood fat.	Date.	Body weight.	Blood sugar.	Blood fat.	Date.	Body weight.	Blood sugar.	Blood fat.	Date.	Body weight.	Blood sugar.	Blood fat.	Date.	Body weight.	Blood sugar.	Blood fat.
Dog 3. Starvation period.				Dog 4. Starvation period.				Dog 1. Starvation period.				Dog 2. Starvation period.				Dog 5. Starvation period.			
1915	*kg.*	*per cent*	*per cent*	*1915*	*kg.*	*per cent*	*per cent*	*1915*	*kg.*	*per cent*	*per cent*	*1915*	*kg.*	*per cent*	*per cent*	*1915*	*kg.*	*per cent*	*per cent*
Nov. 29	11.1	*0.13	*1.01	Nov. 16	6.3	*0.12	*0.91	Nov. 29	9.3	*0.13	*0.71	Nov. 16	6.3	*0.12	*0.91	Nov. 8	16.0	*0.15	*0.69
" 30	11.0	0.11	0.84	" 17	6.2	0.11	0.81	" 30	9.2	0.17	0.69	" 17	6.2	0.11	0.81	" 9	15.6	0.14	0.72
Dec. 1	10.8	0.16	0.84	" 18	6.0	0.12	0.77	Dec. 1	9.0	0.12	0.67	" 18	6.0	0.12	0.77	" 10	15.1	0.13	0.74
" 2	10.6	0.13	0.90	" 19	5.9	0.13	0.80	" 2	8.9	0.13	0.71	" 19	5.9	0.13	0.80	" 11	14.8	0.14	0.74
" 3	10.4	0.14	1.02	" 20	5.8	0.12	0.84	" 3	8.8	0.12	0.80	" 20	5.8	0.12	0.84	" 12	14.6	0.11	0.79
" 4	10.2	0.12	1.05	" 21	5.7	0.11	0.95	" 4	8.6	0.13	0.83	" 21	5.7	0.11	0.95	" 13	14.4	0.12	0.87
Dog 3. Hydrazine period.				Dog 4. Hydrazine period.				Dog 1. Hydrazine period.				Dog 2. Hydrazine period.				Dog 5. Hydrazine period.			
1915				*1916*				*1915*				*1915*				*1915*			
Oct. 24	8.2	†0.12	†1.12	Feb. 1	6.3	†0.12	†0.79	Oct. 11	8.0	†0.13	†0.73	Oct. 18	6.6	†0.11	†0.90	Dec. 6	15.1	†0.14	†0.74
		0.16	1.15			0.12	0.81			0.12	0.74			0.13	0.79			0.15	0.84
" 25	7.6	0.18	1.21	" 2	5.8	0.08	0.91	" 12	7.5	0.15	0.82	" 19	6.2	0.12	0.81	" 7	14.2	0.12	1.09
		0.15	1.72			0.03	0.99			0.15	0.90			0.05	0.96			0.06	1.26
" 26	7.5	0.03	2.04	" 3	5.7	0.04	1.13	" 13	7.3	0.08	0.94	" 20	6.2	0.03	1.21	" 8	14.1	0.04	1.40
		0.02	1.90			0.12	1.01			0.06	0.94			0.02	1.30			0.11	1.21
" 27	7.4	0.12	1.71	" 4	5.7	0.13	0.92	" 14	7.1	0.05	1.04	" 21	6.0	0.15	1.11	" 9	13.9	0.16	1.08
		0.13	1.25			0.12	0.95			0.03	0.86			0.11	1.07			0.11	0.93
" 28	7.2	0.11	1.14	" 5	5.6	0.12	0.83	" 15	7.1	0.08	0.79	" 22	5.9	0.12	1.01	" 10	13.7	0.12	0.95
										0.14				0.13	0.95			0.12	0.98
								" 16	6.9	0.13	0.77	" 23	5.8	0.11	0.95				
										0.12	0.81								

* 24 hr. periods.

† 12 hr. periods.

an increase of blood fat at first followed by a decrease, or else there was no increase.[3] Our animals were taken at random but were all in a well fed condition. The agreement in blood fat of the different animals is fairly close and tends to confirm Bloor's statement that blood fat is quite constant for the species. There seems to be little or no relation between blood fat and blood sugar content during the period of starvation studied.

In the hydrazine period *blood fat shows an increase to a maximum which is coincident with the condition of hypoglycemia induced by hydrazine.* As blood sugar content regains the normal, blood fat assumes a value approximating that of the fasting period.

Whether blood sugar content regulates the blood fat or whether both sugar and fat in the blood are controlled by some other mechanism must be determined by future investigation.

SUMMARY.

In the first days of fasting there is a tendency for the blood fat of dogs to be decreased. This period is followed by one in which there is an increase of fat content to approximately the initial value.

There seems to be little or no relation between blood fat and blood sugar content during the interval of inanition studied.

In hydrazine poisoning blood fat is markedly increased, the maximum being coincident with the condition of hypoglycemia characteristic of hydrazine. Blood fat returns to the fasting value as the sugar of the blood regains the normal.

THE NUTRITIVE VALUE OF YEAST, POLISHED RICE, AND WHITE BREAD, AS DETERMINED BY EXPERIMENTS ON MAN.

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Heretofore the vitamines in foods have been disregarded in all nutrition experiments on man.

The experiments performed on four normal men, as described in this paper, were undertaken with the view to determining primarily the food value of a vitamine-free diet as compared with one containing vitamines. It was found, however, that to insure reliable results the experiment should be carried over a much longer period of time than we were able to devote to it, but as metabolism experiments on man fed on a diet of such simple composition, and under such exact conditions, are so unusual this contribution may be of value. A survey of the work of the past 20 years was covered during this experiment, and we find, where the problem of a protein minimum is considered, that there are two distinct theories.

Abderhalden (1) and his school claim that, as the composition of the body, and especially of the blood proteins, is so different from food proteins, the best protein minimum can be obtained by feeding proteins of the same species.

Hindhede (2), on the contrary, was able to show that when using potatoes as practically the only food, 1 gm. of the absorbed potato nitrogen is equivalent to 1 gm. of body nitrogen. The experiments of the latter author are carried over such a long period that they can be regarded as entirely conclusive. One cause of discrepancy of opinions on this question is that the importance of vitamines in metabolism has been overlooked in most of the more recent experiments. It was pointed out some time ago by one of us (3) that when vitamines are absent in the food mixture,

all animal organisms supplied with a complete food, especially as regards all the necessary amino-acids, will in spite of this come into a negative balance if the experiment is conducted sufficiently long. Hardly any workers in the field of metabolism have realized the importance of this vitamine factor, and some of the conclusions as to the adequacy of proteins or protein mixtures may be erroneously attributed to the incompleteness of the protein. In numerous metabolism experiments on dogs Abderhalden (1) and his school have disregarded the vitamine factor entirely. They often compare a food containing artificially digested meat and vitamines with a food mixture deficient in these latter ingredients. Hindhede (4), on the contrary, takes this vitamine factor into account and gives a potato diet on which no deficiency diseases occur (5). Studying the earlier literature on protein minimum we find that the work of Munk (6) and of Rosenheim (7) gives a typical illustration of the point we wish to emphasize. Dogs which they used in their experiments were given both rice and meat powder, but they overlooked the fact that the greater part of the vitamines was lost by drying and heating. The authors gradually decreased the meat in the diet and replaced it by more carbohydrates in the form of rice. As the result of this procedure the dogs showed a negative nitrogen balance and some of them died of the effects described by Schaumann (8) when he fed them on extracted meat; the others recovered when fresh meat was added.

We know now through the work of one of us (9) and of Braddon and Cooper (10) that the amount of carbohydrates in the diet cannot be increased indiscriminately, as a certain amount of vitamine can take care of only a limited amount of carbohydrates. In other words, there must be a fixed ratio between the vitamine content and the carbohydrate. If this ratio is destroyed, disordered nutrition of the tissue cell occurs. The conclusions of Munk and Rosenheim that dogs require a large supply of protein are erroneous, as some years ago Funk performed metabolism experiments in which dogs were readily brought into nitrogen equilibrium with meat powder or milk powder, while difficulties were encountered when they were fed on edestin or gliadin. In one of the experiments of Abderhalden and Funk (11), where comparative studies were made as to the value of edestin and gliadin, a great number of the dogs died. In one case a

nitrogen equilibrium was established for a short period with gliadin but, notwithstanding, the dog steadily lost weight. We do not wish to minimize the importance of aromatic amino-acids, cystine, trytophane, and lysine, but we wish to point out that many of the experiments on the necessity of certain amino-acids should be repeated by taking the vitamine factor into account.

In studies on protein minimum this latter condition should also receive consideration. In a recent paper of Abderhalden, Fodor, and Röse (12) we find a comparative study of the food value of potatoes as compared with whole bread and white bread. The protein minimum was lowest in the case of potatoes (4.5 gm. nitrogen), higher with whole meal bread (7.0 gm. nitrogen), and still higher with white bread (9.+ gm. nitrogen), a possible reason being that the white bread was deficient in vitamines.[1]

EXPERIMENTAL.

Four of the members of the laboratory staff volunteered their services in the feeding experiments with yeast, white rice, and bread, and continued their duties during the whole experimental period. Our diet before the experiment was of normal variety and quantity, in contrast to former investigations, in which the subjects even before the experiment were accustomed to eat food without change or variety. The attempt was made to live on yeast[2] as the only source of nitrogen. During the preliminary period the amount of nitrogen in the excreta was studied under normal dietary conditions. The necessary amount of calories was then supplied in the form of fruit and vegetables. These were chosen to contain the minimum amount of nitrogen and a still smaller amount of protein nitrogen.

Although yeast has been extensively used for feeding cattle for a number of years, its use for human consumption has been suggested only recently in Germany, owing to the scarcity of meat. As a meat substitute it was strongly recommended by Schottelius (13), who has used it in quantities of 50 to 75 gm. daily in addition to the ordinary diet, and he claims that it is well utilized in

[1] Averages in feces were: On potatoes 0.4 to 0.7 gm. nitrogen, in whole meal bread 1.6 to 1.9, and in the case of white bread 1.5 gm. nitrogen. The lower value of white bread is therefore not due to faulty assimilation.

[2] Yeast used by us was a dried anaerobic yeast preparation which was put at our disposal by the American Pure Yeast Company of New York.

the human body. Before undertaking our experiments we tried the availability of yeast in rats, substituting it for casein nitrogen. The animals, records of which will be published later, show that *young rats can live on yeast as the sole nitrogen source for quite a long while, although it has not yet been proven whether they can subsist on it indefinitely.* The experiment showed that yeast contains a protein apparently complete as to its composition in amino-acids, corroborating Neuberg's work (14) on the proteins of yeast. But there was some difficulty in determining how much of the yeast nitrogen is represented by protein nitrogen. Neuberg, by a complicated method, ascertained that yeast protein somewhat resembles casein, and amounted in Schroeder's yeast to 48.3 per cent of the total nitrogen, whereas the bottom fermentation yeast K, from the Institute for Brewing in Berlin, was found to contain only 46.3 per cent. Bokorny (15) states that yeast in a dried state contains 60 per cent protein. We also attempted to determine the protein content of our yeast and found that the usual method of determination, as used by Stutzer, failed completely. This method showed that about 90 per cent of the yeast nitrogen was protein nitrogen. Finally, we tried to estimate the yeast nitrogen indirectly by determining the purine base nitrogen, and for this determination followed the method of Burian and Schur (16), hydrolyzing the yeast with dilute sulfuric acid and precipitating the purine bases in an alkaline solution, with sodium bisulfite and copper sulfate, then estimating the nitrogen in the copper precipitate. In this way we recovered 13 per cent of the yeast nitrogen as purine bases, which undoubtedly was too low a figure.

The literature records only a few actual metabolism experiments with yeast, one of Praussnitz and Menicanti (17), and another of Thomas (18), who, while experimenting with other foodstuffs, prepared a pressed yeast with butter, and used it for 3 days. The nitrogen balance in this case was negative with over 4 gm. of nitrogen in the form of yeast. The yeast was well absorbed as shown by the feces nitrogen, which was rather low.

The utilization of yeast was insufficient in our experiment where yeast was taken for nearly a fortnight, as the nitrogen content of feces was high, and in some instances undigested yeast was detected. With two out of four persons a practically posi-

tive balance was obtained for several days. The experiments were performed in the beginning of June, when cold weather prevailed and some difficulty was experienced in maintaining bodily heat. In spite of the small food supply there was no craving for food. The bowels moved with regularity and no diarrhea occurred. The bad utilization of yeast may be partially due to the fact that the yeast had a disagreeable taste and was unpleasant to eat for any length of time.

During the second period the diet consisted of white rice, white bread, and butter. We had very much less nitrogen in this form than Kumagawa (19), who weighed 49 kg., and took 600 gm. rice, 100 gm. Miso (a fermented mixture of rice and beans), 10 cc. soja, 600 cc. beer, 28 gm. sugar, 300 gm. of turnip-cabbage, and 600 cc. tea, averaging 8.75 gm. nitrogen per day. Our vitamine- and purine-free diet was supplemented in the later period by vitamine from 50 cc. of autolyzed yeast, prepared according to the method of Seidell (20), using Lloyd's reagent, a colloidal aluminum silicate preparation of unknown composition.

In one case which was kept as a control, the vitamine was omitted. We were unable to find that upon its addition the balance became more positive. The reason for this may be ascribed to the shortness of the experiment, or to the fact that the vitamine preparation, made in the above mentioned way, was not sufficiently active.

An added interest in our metabolism experiments was the chemical examination of blood. The negative balance found is partially due to this, and although the nitrogen lost in this way was determined, it was not included in the balance, as the blood nitrogen is partially composed of food nitrogen already assimilated, and partially of nitrogen which would be subsequently eliminated through the kidney or through the intestinal tract. The loss, however, is added in the blood analyses. On the days of the chemical blood examinations, a partition of the different nitrogenous constituents of the urine was also determined. This had been done once before in a metabolism experiment by Folin (21), but in his case the nitrogen intake was held to normal limits (about 16 gm.). In a few instances on the vitamine-free diet the sugar in the urine was determined by Bertrand's method. Although the blood sugar was not more than usual, the urinary

sugar was much higher than in normally fed cases, as determined by the same method by Funk (22). This may be due to alimentary glycosuria following a high carbohydrate diet, or may be analogous to the findings of one of us with Schönborn (23) in pigeons, where a considerable increase in blood sugar was found on a rice diet. We shall soon begin to investigate this problem in diabetics on vitamine-free and vitamine-containing diets.

The nitrogen content of foodstuffs consumed by us was determined actually in all instances. The feces at the beginning of the feeding experiment were marked by feeding charcoal. In the second period this was not necessary, as the color changed from brown to light yellow. On resuming a normal diet diarrhea developed in three out of the four cases.

TABLE I.

Nitrogen Contained in the Foodstuffs Consumed During the Experiment.

	per cent		*per cent*
Dried yeast	9.53	Banana	0.31
Onion yellow	0.31	Sweet chocolate	0.071
Sauerkraut	0.277	Tea biscuit	1.41
Butter	0.055	Bread (baker's)	1.27
Apple	0.096	Tea, 3 gm. to 800 cc	0.008
Tomato soup	0.68	Tomato	0.71
Rice	1.21	Orange	0.282
Yeast vitamine, 50 cc. autolyzed	0.078		

Case I.—A normal vigorous man, 38 years old, weighing 78 kg. at the beginning of the experiment.

Diet (Gm.).

Yeast period.

Days.	1	2	3	4	5	6	7	8	9	10	11	12
Yeast	30	30	50	60	60	60	60	60	60	60	60	60
Apple	482	482	480	1,045	514	378	250	247	402	277	293	280
Banana	101	127	123	84	74	167	145	91			182	114
Chocolate	21	63	63	63	126	63	130	63	94		94	336
Tea, cc	300	300	600	300	300	300	900	600	300			600
Butter							55	49			25	
Onion			71	127	192	161	98	68	150			118
Orange							110			131	124	156
Sauerkraut								100				
Tomato								65				
Sugar	14	7	28	14	7							

Rice and bread period.

Date.	1	2	3	4	5	6	7	8	9	10	11	12
Bread.....			238	163	237	237	238	238	238	....	....	...
Tea biscuit.....	204	183									....	...
Butter.....	100	50	62	87	52	74	115	98	114	....	....	...
Rice.....	200	200	200	200	200	200	200	200	200	....	....	...
Water, cc.....	3,000	1,776	604	1,031	750	891	779	851	694	....	....	...

Metabolism Experiment, Case I.

Date.	N intake.	N output.	N in urine.	N in feces.	Urine.	Feces.	Weight.	Balance.	Remarks.
Normal diet.									
1916	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*kg.*		
June									
3		11.38	10.10	1.28	1,700	33.5	79.4		
4		10.35	9.05	1.30	1,740	45.0	78.9		
5		10.35	9.05	1.31					
Yeast period.									
6	3.60	8.06	6.70	1.36	820	26.0	78.0	−4.46	
7	3.71	11.58	10.95	0.63	1,840	21.0	77.4	−7.24	
8	5.92	11.24	10.61	0.63	1,540		77.4	−5.32	
9	7.97	9.86	7.06	2.80	1,180	52.0	77.1	−1.89	Blood taken.
10	7.21	8.51	6.79	1.72	1,120	33.7	77.1	−1.31	
11	7.13	8.02	6.92	1.10	2,160	30.0	76.6	−0.89	
12	7.34	7.94	6.84	1.11	1,350		76.6	−0.60	Blood taken.
13	7.89	9.81	8.05	1.76	1,270	25.0	76.6	−1.92	
14	6.66	6.10	5.53	0.57	500	20.0	76.4	+0.56	Blood taken.
15	6.47	9.74	9.16	0.58	1,450		75.7	−3.26	
16	6.99	13.62	9.88	3.74	1,760	66.0	75.2	−6.63	Blood taken.
17	7.34	9.76	8.19	1.57	2,120	24.7	75.0	−2.42	
Rice and bread period.									
18	5.35	5.96	4.39	1.57	380	24.7	76.6	−0.61	
19	5.03	9.40	7.22	2.18	730		77.1	−4.37	
20	5.47	10.12	7.94	2.18	1,450		76.2	−4.65	
21	4.54	8.19	7.42	0.77	1,100		74.6	−3.64	Blood taken.
22	5.45	7.54	6.77	0.77	780	58.0	75.0	−2.00	
23	5.47	6.52	5.75	0.77	460		74.8	−1.05	
24	5.50	8.04	7.27	0.77	660		74.5	−2.54	
25	5.49	5.64	4.87	0.77	340		74.8	−0.15	
26	5.50	6.98	6.21	0.77	400		75.9	−1.48	Blood taken.

Urine Analysis, Case I.

Date	Ammonia.	Urea.	Creatinine.	Uric acid.	Volume.	Sugar
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*
9	0.510	10.6	1.04	0.895	1,120	
12	0.504	10.6	1.57	0.90	1,350	
14	0.304	9.95	0.826	0.53	500	
16	0.790	15.4	0.890	1.32	1,760	
20					1,450	2.10
21	0.623	7.55	1.47	0.469	1,100	1.62
23					460	1.52
24					660	1.78
26	0.024	10.3	1.28	0.388	400	

Blood Analysis, Case I.

Date.	Total N.	Non-protein.	Urea N.	Creatinine.	Uric acid.	Sugar.	CO_2	Volume.	Remarks.
June	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*		*cc.*	
9	3.26	25.4	23.8	0.75	4.0	0.108	46	55	Yeast diet.
12	3.30	29.4	28.5	0.57	2.0	0.106	47	65	
14	5.04	32.7	34.6	0.72	4.2		46	55	
16	3.22	25.1	27.7	0.85	5.0	0.092	45	54	
21	2.87	25.8		1.3	2.8	0.090	41	48	Rice diet.
26	2.57	28.5	32.8	2.2	3.5	0.091	49	41	
Total loss of nitrogen by bleeding.	10.78								
July 7	2.84	37.8	29.4	0.5	1.3	0.0744			Normal diet.

In considering the results of the metabolism experiment in Case I we find that yeast was fairly well utilized in the middle of the first period, but later was found, to some extent, undigested in the feces. However, in the period in which white rice and bread were given as a control for a vitamine-free diet, the man under experiment went more and more into nitrogen balance without the addition of vitamine. This might be due to the fact that less water was consumed in the latter part of the experiment. A high uric acid content occurred in the blood in the yeast period, and a fairly high sugar content in the urine on the vitamine-free diet.

Case II.—A normal man, 22 years old, weighing 68.4 kg. at the beginning of the experiment.

Diet (Gm.).

Yeast period.

Days	1	2	3	4	5	6	7	8	9	10	11	12	13
Yeast	25	25	35	50	50	50	55	55	55	55	55	55	55
Apple	145.5	434	211	173.5	221	358				207		98	73
Banana	106.1	155	258.5	118			91.5	87.5				117	
Chocolate		21	84	52.5	30.5	94.5		126	84	73.5	115.5	63	
Tea, cc	300	750	450	450		600		300					
Butter			50			10							
Onion			102				127						
Orange							152				96	79	142
Sauerkraut								150	100	100	100		
Tomato soup		54		50	40	100		62	62	59	62	62	
Sugar	40	24	32	32	24	32	32	24					
Bread										4			75

Rice and bread period.

Bread	228	228	228	228	228	228	228	228					
Butter	100	100	100	100	100	100	100	100					
Rice	175	175	175	175	175	175	175	175					
Water, cc	700	700	700	700	700	700	700	700					
Yeast, vitamine, as colloidal silica preparation					4.8	4.8	4.1	3.0					

Metabolism Experiment, Case II.

Date.	N intake.	N output.	N in urine.	N in feces.	Urine.	Feces.	Weight.	Balance.	Remarks.
Normal diet.									
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*kg.*		
3		16.29	15.96	0.33	1,125		69.1		
4		16.27	15.94	0.33	1,260	17.9	69.1		
5		14.19	13.86	0.33	1,140		68.9		
Yeast period.									
6	4.46	11.25	9.82	1.43	900	23.3	68.4	−7.28	
7	3.97	9.02	7.03	1.99	640	33.0	68.2	−3.96	
8	5.09	8.78	6.68	2.1	950	37.5	67.9	−3.69	
9	5.77	8.19	6.87	1.32	720	38.0	67.0	−2.42	Blood taken.
10	5.90	9.33	8.01	1.32	840		67.0	−3.43	
11	5.90	8.46	7.04	1.42	800	36.0	66.4	−2.56	
12	6.16	7.63	6.20	1.43	1,210		65.7	−1.47	Blood taken.
13	6.25	7.61	6.38	1.23	540	40.0	65.2	−1.36	
14	6.04	8.04	6.80	1.24	545		65.2	−2.00	Blood taken.
15	6.24	6.73	5.65	1.08	600	56.0	65.3	−0.49	
16	6.10	5.99	4.91	1.08	575		65.3	+0.11	Blood taken.
17	6.40	7.57	6.59	0.98	1,020	51.5	65.8	−1.17	
18	6.77	6.57	5.58	0.99	1,250		65.3	+0.20	
Rice and bread period.									
19	5.07	7.68	6.80	0.88	800	Feces	65.0	−2.61	
20	5.07	6.91	6.03	0.88	560	for 4	65.0	−1.84	
21	5.07	6.69	5.81	0.88	525	days	64.6	−1.62	Blood taken.
22	5.07	7.68	6.80	0.88	640	48.5	65.3	−2.61	
Vitamine addition.									
23	5.15	6.56	5.69	0.87	480	51.4	64.6	−1.41	
24	5.15	6.49	5.62	0.87	680		64.6	−1.34	
25	5.15	7.46	6.59	0.87	750		65.2	−2.31	
26	5.15	6.18	5.31	0.87	490		65.0	−1.03	

Urine Analysis, Case II.

Date.	Ammonia.	Urea.	Creatinine.	Uric acid.	Volume.	Sugar.
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*
9	0.354	10.10	1.50	0.604	720	
12	0.348	9.85	1.60	0.930	1,210	
14	0.402	12.10	1.08	0.410	545	
16	0.390	6.32	0.725	0.569	575	
21	0.370	7.54	0.894	0.620	525	1.89
24					680	1.56
25					750	1.78
26	0.417	7.70	1.13	0.475	490	

Blood Analysis, Case II.

Date.	Total N.	Non-protein.	Urea N.	Creatinine.	Uric acid.	Sugar.	CO_2	Volume.	Remarks.
June	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*		*cc.*	
9	3.26		26.3		3 5			15	
12	2.78	29.8	26.3	0.24	4.0	0.111	45	83	Yeast diet.
14	5.66	30.2	22.1	0.86	8.0	0.100	46	50	
16	3.20	23.7	21.0	1.20	4.8	0.099	46	56	
21	2 65	26.5		0.51	2.0	0.100	41	71	Rice diet.
26	2.72	27.0	29.4		1.5	0.097	43	32	
Total loss of nitrogen by bleeding.....	10.17								
July									
7	2.46	30.0	24.6	0.7	1.7	0.110	48		Normal diet.

In Case II the noticeable factors are the fairly good nitrogen balance obtained, and the high figures for uric acid in blood with yeast. Vitamine gave no definite results in the rice period.

Case III.—A normal man, 20 years old, weighing 65.7 kg. at the beginning of the experiment.

Diet (Gm.).

Days.	1.	2	3	4	5	6	7	8	9	10	11
Yeast period.											
Yeast	35	35	45	50	50	55	55	65	55	60	60
Apple	243	138	111	110	86	326	79	404	310	121	
Banana	83	128	138	193	90			81	100	77	78
Chocolate	31.5	63	126	126	273	168	318	147	210	231	136.5
Tea, cc.	600	900	900	900	600	1,200	1,400	900	900	900	900
Butter	173	162	99	50	10	60	50				
Onion			70	76							
Orange						152					
Tomato soup				50		100	45				
Tomato										59	63
Bread	50	50			50		50				
Sugar	28	14	49	49	28	80	66	52	49	42	63
Rice and bread period.											
Bread	200		200	200	210	200	200	200	200	200	
Tea biscuit		185									
Butter	100	100	105	129	110	120	120	120	120	120	
Sugar					25		25	2.	25	25	
Water, cc.	1,163	1,190	761	1,193	1,308	1,211	954	1,011	1 055	612	
Vitamine						3.75	4.81	4.8	4.16	3.0	

Rice 155 gm. throughout each day of the whole period.

Metabolism Experiment, Case III.

Date.	N intake.	N output.	N in urine.	N in feces.	Urine.	Feces.	Weight.	Balance.	Remarks.
Normal diet.									
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*kg.*		
3		15.38	14.43	1.90	1,000	29.6	65.0		
4		13.95	13.0	1.90	1,020		65.0		
5		13.83	13.37	0.46	1,620		65.0		
Yeast period.									
6	3.63	10.96	10.49	0.47	700	12.8	65.7	−7.33	
7	4.64	11.46	10.47	0.99	600	19.1	65.4	−6.82	
8	4.62	10.18	9.27	0.91	1,140	17.3	64.8	−5.56	
9	6.21	9.97	8.15	1.82	1,000	30.9	64.4	−3.76	Blood taken.
10	6.02	11.88	8.38	3.50	670	34.0	63.8	−5.86	
11	7.00	9.20	7.32	1.88	1,040	62.49	64.1	−2.20	
12	6.63	10.22	8.33	1.89	1,525		63.5	−3.59	Blood taken.
13	6.62	7.53	6.04	1.49	600	45.7	63.7	−0.91	
14	6.07	8.72	7.23	1.49	620		63.2	−2.65	
15	6.72	10.45	7.56	2.89	780	50.2	63.9	−3.73	
16	7.00	13.54	7.48	6.06	650	101.8	63.1	−6.54	Blood taken.
Rice and bread period.									
17	4.47	8.06	7.18	0.88	550	44.9	63.9	−3.59	
18	4.54	8.79	7.91	0.88	780		63.7	−4.25	
19	4.47	8.30	7.42	0.88	780		63.2	−3.83	
20	4.48	7.82	7.56	0.26	720		63.0	−3.34	
21	4.60	7.28	7.02	0.26	500		62.9	−2.68	Blood taken.
Vitamine addition.									
22	4.56	5.39	5.13	0.26	580	26.2 including June 20 and 21.	63.2	−0.83	
23	4.56	6.06	5.80	0.26	520		62.8	−1.50	
24	4.56	7.01	6.75	0.26	510		63.1	−2.45	
25	4.56	7.94	7.68	0.26	1,020		63.2	−3.38	
26	4.56	6.87	6.61	0.26	1,040		63.0	−2.31	

Urine Analysis, Case III.

Date.	Ammonia.	Urea.	Creatinine.	Uric acid.	Volume.	Sugar.
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*
9	0.500	11.6	1.6	0.875	1,000	
12	0.412	22.7	1.64	1.18	1,525	
16	0.396	10.5	0.908	0.487	650	
21	0.452	7.45	0.815	0.405	500	0.41
26	0.420	10.1	1.65	0.641	1,040	

Blood Analysis, Case III.

Date.	Total N.	Non-protein N.	Urea N.	Creatinine.	Uric acid.	Sugar.	CO_2.	Volume.	Remarks.
June	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*		*cc.*	
9	3.02	31.2	25.6	0.59	1.8	0.107	49	55	
12	3.18	28.8	25.7	0.25	1.9	0.104	49	64	Yeast diet.
16	3.08	31.8	27.2	0.13	4.9	0.084	47	Lost.	
21	2.66	28.0		0.59		0.088	46	61	Rice diet.
26	2.87	29.0	27.2	2.1	3.0	0.086	45	56	
Total loss in nitrogen by bleeding.....	8.4								
July									
7	2.58	33.0	39.6	0.6	2.8	0.119	46		Normal diet.

Case IV.—A normal man, 32 years old, weighing 63.5 kg. at the beginning of the experiment.

Diet (Gm.).

Yeast period.

Days.	1	2	3	4	5	6	7	8	9	10	11
Yeast.....	20	20	40	50	50	55	55	55	55	60	50
Apple.....	275	350	328	187	152	323	390		180	95	105
Banana....	202	205	132	121	173	74	73	93			
Chocolate..	84	157.5	84	147	168	147	92	107	126	168	148
Tea, cc.....	300	300	900	1,200	1,500	1,350	1,200	1,800	1,500	950	1,100
Butter.....			30	41			25				
Onion.....			10	25			14				
Orange.....					110	100	95		350	70	50
Sauerkraut.								124	250	100	200
Sugar.....	14	11	28	60	70	66	58	72	56	56	63
Water, cc..										150	150

Rice and bread period.

Bread.....	200		200	200	200	200	200	200	200	200
Tea biscuit.....		185								
Butter.....	100	100	100	100	110	115	110	110	110	110
Water, cc..	1,250	1,155	639	680	891	1,066	821	916	854	673
Vitamine as Lloyd's reagent...						3.75	4.8	4.8	4.1	3.0

Rice 155 gm. throughout the period.

Metabolism Experiment, Case IV.

Date.	N intake.	N output.	N in urine.	N in feces.	Urine.	Feces.	Weight.	Balance.	Remarks.
				Normal diet.					
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*kg.*		
3		13.07	11.26	1.81	915	31.05	64.1		
4		12.75	11.2	1.55	760	25.3	64.1		
5		13.81	11.0	2.81	745	45.25	64.1		
				Yeast period.					
6	2.91	11.02	9.85	1.17	1,440	18.8	63.5	−8.11	
7	3.03	8.03	6.4	1.63	700	24.4	62.7	−5.00	
8	4.70	10.67	8.23	2.44	840	36.1	62.3	−5.97	
9	5.62	9.54	7.88	1.66	870	28.5	61.8	−3.94	Blood taken.
10	5.97	12.83	8.22	4.61	1,180	72.65	61.9	−6.88	
11	6.28	10.36	8.23	2.13	1,160	62.75	61.5	−4.08	
12	6.31	8.99	6.85	2.14	825		61.2	−2.68	Blood taken.
13	6.00	8.93	7.21	1.72	740	50.9	61.3	−2.93	
14	6.41	9.55	7.83	1.72	660		61.3	−3.13	Blood taken.
15	6.48	10.74	8.76	1.98	770	58.85	61.3	−4.26	
16	5.75	9.55	7.57	1.98	630		61.0	−3.80	Blood taken.
				Rice and bread period.					
17	4.47	6.90	6.09	0.81	420		61.2	−2.43	
18	4.54	7.08	6.27	0.81	480		61.8	−2.54	
19	4.47	6.44	5.63	0.81	440	56.0	61.5	−1.97	
20	4.47	6.18	5.37	0.81	530		61.3	−1.71	
21	4.48	5.74	4.93	0.81	460		60.9	−1.26	Blood taken.
				Vitamine addition.					
22	4.56	5.08	5.08	0.72	410		61.1	−1.24	
23	4.56	5.58	4.86	0.72	430		61.1	−1.02	
24	4.56	6.23	5.51	0.72	405	54.0	61.2	−1.67	
25	4.56	6.34	5.62	0.72	490		61.1	−1.78	
26	4.56	6.63	5.91	0.72	480		60.7	−2.07	Blood taken.

Urine Analysis, Case IV.

Date.	Ammonia.	Urea.	Creatinine.	Uric acid.	Volume.	Sugar.
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*
9	0.354	12.2	1.20	0.90	870	
12	0.335	10.6	1.25	0.75	825	
14	0.370	12.8	1.29	0.533	660	
16	0.432	11.6	0.645	0.497	630	
21	0.410	7.14	1.29	0.409	460	0.27
24					405	0.54
26	0.452	9.2	1.20	0.465	480	

Blood Analysis, Case IV.

Date.	Total N.	Non-protein N.	Urea N.	Creatinine.	Uric acid.	Sugar.	CO_2.	Volume.	Remarks.
June	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*		*cc.*	
9	4.30	34.0	33.6	1.1	4.8	0.119	39	60	
12	3.90	29.4	31.2					9	Yeast diet.
14	3.16	27.2	43.0	0.62	5.9		47	60	
16	3.18	30.2	34.8	1.1	5.6	0.093	49	60	
21	2.92	32.2	29.0	2.2		0.091	43	58	
26	2.59	30.6	33.6	1.1	2.9	0.105	46	67	Rice diet.
Total nitrogen lost by bleeding..	10.16								
July 7	2.79	32.3	40.3	1.3	1.9	0.12	48		Normal diet.

Besides the blood analysis the blood pressure was taken June 13, and found to be as follows: Case I, 125 mm.; Case II, 130 mm.; Case III, 115 mm.; and Case IV, 120 mm.

Besides the blood pressure the hemoglobin content and also a differential leukocyte count were registered on June 16 on the yeast diet, which figures were found normal:

Case.	Hemoglobin.	Polymorphonuclears.	Leukocytes.	Large mononuclears.	Transitionals.	Eosinophils.	Basophils.
I	88	42	50	1	1	5	1
II	89	69	29	1	1	0	0
III	80	65	32	0	1	1	0
IV	82	66	33	0	0	1	0

As a result of our nutrition experiments with yeast it is shown that the value of yeast as a protein source is not very great. Thomas (18), by comparing the protein substitution value of different foodstuffs, concluded that the biological value of yeast is 70.52 per cent. From our experiments this is certainly not the case, as a large quantity of unutilized yeast passes in the feces and is lost. Yeast, on account of its high purine content, causes a distinct rise of uric acid in the blood, and for this reason cannot be used to the exclusion of all other food. Our experiments with vitamine-free food, in the form of white rice and white bread, confirm the results of Abderhalden, Fodor, and Röse, who found that to obtain a positive nitrogen balance, more white bread is required than whole bread or potatoes. In their experiments as well as in ours, this negative balance occurred in spite of the low nitrogen figures in the feces, and indicated good resorption. Whether this low value of vitamine-free food is due solely to the absence of vitamines, we were not able to prove as our experiments were of such comparatively short duration.

RÉSUMÉ.

Yeast cannot very well be recommended as a sole protein source, as a large part of the yeast nitrogen apparently has no food value. It is badly assimilated and occasions a rise of uric acid figures in the blood. The amount of nitrogen which would be fully adequate in the form of potatoes was proved to be insufficient with yeast. This also applies to our experiment with white bread and white rice. We were unable in our experiment to get a positive nitrogen balance by the addition of vitamine.

In this field of nutrition research our studies have by no means enabled us to pronounce a verdict that yeast possesses no value in dietetics.

Problems of personal idiosyncrasy as to the taste of foods, methods of administration suitable for the particular individual, questions of amino-acid synthesis after the yeast protein and purine bases have entered the organism, are all complex factors which must be taken into consideration.

Just how far anaerobic yeast, when consumed in conjunction with other foods, can be made of any supplementary metabolism

value to a large community suffering from dietetic deficiencies of one type or another, offers an inviting field for further research in the domain of practical experimentation.

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THE NUTRITIVE PROPERTIES OF CORN.

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It has long been a matter of common knowledge that an exclusive diet of maize is inadequate for growing animals, but the precise nature of the deficiency is not yet determined. Thus one school of investigators has ascribed the nutritive deficiencies of corn to its lack of certain inorganic constituents. Forbes (1914) states:

"Our results show that, whatever the protein deficiencies of corn, its mineral deficiencies are more pronounced, since, in balance experiments, the deficiencies in calcium and other minerals are immediately made manifest by negative balances or deficient storage, while the protein deficiencies, whatever their nature, allow liberal nitrogen retention.

The extreme importance of the mineral constituents cannot be denied, but some investigators have laid more stress on the protein deficiencies of corn than does Forbes. For example, Osborne (1913) states:

"The results when presented leave no doubt that the deficiency observed in the practical feeding of corn meal is explained largely, if not wholly, by the unique chemical constitution of zein which forms such a large part of its protein."

Another suggestion frequently offered is that the growth accessory substances, the so called "vitamines," are present in such small quantities that normal growth cannot occur unless they are supplied in some supplementary food. As yet, however, there is no convincing evidence at hand to support this theory.

EXPERIMENTAL PROCEDURE.

Most of the data described in this preliminary report were obtained by using albino rats as experimental animals. This procedure has proved so successful at various institutions that

further comment on this point is unnecessary. Our choice, however, is explained by the anticipated use of lysine, tryptophane, and isolated proteins. The cost of these would be prohibitive if prepared in sufficient quantity for any of the ordinary domestic animals. During feeding trials the animals were confined in cages of hardware cloth, following closely the procedure of Osborne and Mendel (1911). Distilled water *ad libitum* was given in all cases.

I. Adequacy of the Inorganic Constituents.

This particular problem was only incidental in the original plan, so our data on this point are relatively meager. Some of the animals were given corn alone, others corn plus a salt mixture. A third lot received corn plus an "ash-free" protein,[1] and a fourth, corn plus "ash-free" protein plus a salt mixture. The results are given in Table I.

The results at least have a positive trend. If corn is the sole article of diet the animals decline rapidly in weight and soon die. The addition of a salt mixture,[2] however, permitted growth to occur in a surprising degree. When an "ash-free" protein is added to the corn, in most cases death ensues promptly. The dried blood is a striking exception. At the end of 27 weeks the animals were apparently growing slowly. Possibly they would

[1] The author prepared these materials originally for another research conducted jointly by the executive, chemistry, and animal husbandry departments of the college. The methods of preparation are described later in this article.

[2] Two salt mixtures were used, apparently with equal success. No. 1 was adapted by Dean Willard, of this college, from a mixture published by Osborne and Mendel. No. 2 approximates a mixture published by McCollum.

	No. 1 gm.	No. 2 gm.
$CaCO_3$	92	
Ca lactate	8	468.0
$Ca_3(PO_4)_2$	10	
K_2HPO_4	37	280.8
NaCl	20	123.1
Na citrate	15	31.2
Fe citrate	2	23.8
$MgSO_4$		31.72

TABLE I.

Growth (Gm.) of Rats on Rations of Low and High Mineral Content.

	Corn alone.			Corn + salt mixture.			Corn + ash-free egg white.		Corn + ash-free dried blood.		Corn + corn gluten.		Corn + ash-free casein.		Corn + ash-free egg white + salt mixture.			Corn + dried blood + salt mixture.		Corn + corn gluten + salt mixture.		Corn + ash-free casin + salt mixture.	
No. and sex.	♀ 59	♀ 58	♂ 60	♀ 60	♂33	♀ 34	♀ 61	♂ 62	♀ 37	♂ 38	♂ 63	♀ 64	♀ 65	♂ 66	♂67	♀ 29	♀ 30	♀ 35	♂36	♀ 68	♀ 69	♂70	♀ 71
Initial weight.	57	57	55	89	48	45	50	52	53	57	47	42	53	89	56	70	62	48	57	45	35	47	50
wks.																							
3	59	58	60	95	65	64	64	78	95	87	55	47	60	79	85	92	97	60	74	78	65	83	80
6	65	61	62	120	100	100	50	72	100	105	72	52	67	84	107	90	115	93	123	115	105	146	105
9	67	58	63	135	135	115	Dead.	Dead.	107	112	74	Dead.	53	77	132	105	122	90	153	140	138	162	135
12	70	Dead.	65	133	160	135			115	117	75		Dead.	Dead.	177	115	132	127	167	167	148	185	155
15	65		63	140	175	135			110	112	62				180	120	130	125	172	165	150	195	165
18	Dead.		Dead.	133	187	140			115	130	Dead.				175	120	140	125	180	175	156	210	172
21				130	200	145			120	130					215	125	145	130	195	180	167	215	175
24				140	220	160			133	140					225	130	140	135	190	200	180	Diet	
27				145	265	190			135	125					225	Diet		145	195	215	205	changed.	
30				125	275	190			Diet							changed.		145	200	225	220		
33				110					changed.									Diet		Diet			
36				120														changed.		changed.			
39				115																			
				Killed.																			

Rats 33 and 34 were mated Mar. 1, and five young were delivered Apr. 4. All were alive at birth, but with one exception they did not survive long. The mother began eating the young almost immediately after delivery, and so in some cases the young may not have met a natural death. Although numerous litters have been reared in this laboratory, this is the only case observed where any of the young have been eaten.

have declined later, but our facilities would not allow us to continue the trial longer.

A somewhat similar experiment has been described by Weiser (1914). His object was to determine the effect of a calcium-poor diet on the composition of growing bones. One group of three pigs received maize and corn gluten, another group of three received the same ration plus about 0.75 per cent of calcium carbonate. Some time after the feeding trial began dried blood was substituted for corn gluten. During the first part of the trial the weights of the lots only were given, as the individual records are not available. During the latter part of the trial the individual weights are recorded. A portion of the record is summarized in Table II.

TABLE II.

Growth (Kg.) of Swine on Rations of Low and High Calcium Content.

	Lot with Ca.	Lot without Ca.	
Jan. 15	17.7		17.6
June 15	41.8		36.8
	No. 1.	No. 4.	No. 6.
July 7	19.6	14.5	9.9
Sept. 24	35.7	12.6	11.1

Even the animals receiving calcium grew slowly, but the increased growth due to its addition to the diet is quite marked.

Data obtained at this institution are not in complete agreement with Weiser's findings. Extensive feeding trials here have been conducted jointly by the executive, animal husbandry, and chemistry departments, using swine as experimental animals. One lot received corn alone, and another corn plus salt mixture No. 1. The figures are very striking, and as they represent the experience of several years, there can be no doubt that they are entirely trustworthy. On corn alone, the animals can barely maintain life. Some of them in fact do succumb, while a few others maintain life in a precarious condition for some months, but gradually gain in weight, and also show a real increase in length and height. After some months the animals grow more rapidly, they take on a more thrifty appearance, and in the

course of time reach a long deferred maturity. A striking point in these researches is the failure to derive any benefit from the use of a salt mixture. In contrast to this stands the real value derived from the addition to corn of a protein comparatively free from inorganic constituents. Two substances were used for this purpose; the proteins of blood were used in one trial, and those of egg white in the other. Both of these are comparatively free from calcium and phosphorus, but they were given special treatment to remove from them so far as practicable all inorganic constituents. The blood, obtained from Armour and Co., was defibrinated in their plant, chilled, and shipped here. As soon as possible after its receipt it was diluted to four or five times its volume, acidified with acetic acid, and heated to boiling with live steam. The coagulum was filtered off on a linen cloth, returned to the vessel in which coagulation occurred, and washed with a large volume of boiling water. This was again filtered, and dried at 70–80°C. The egg white used was a dried commercial product. This was dissolved in warm water, and then given practically the same treatment as the blood. The analyses are shown in Table III. All animals drank distilled water during the feeding trial.

TABLE III.

Ash and Nitrogen Analyses of Feeds.

	Total ash.	Ca	P	N
	per cent	*per cent*	*per cent*	*per cent*
Corn	1.57	0.015	0.288	1.57
Blood proteins	1.09	0.0126	0.0824	15.21
Egg white	0.57	0.0110	0.089	14.75
Starch	1.02	0.0132	0.196	0.056

As far as growing swine are concerned, therefore, corn contains sufficient inorganic material for the protein already present. It would seem in this case that the first limiting factor is the protein. It is very certain that corn does not contain enough mineral matter for normal growth. In support of this statement additional data are submitted. Other lots of swine received corn and the "ash-free" protein and salt mixture No. 1. In this case growth was approximately normal.

The final results of these feeding trials are given in Table IV. The upper part of the table gives the composition of the feeds in percentages. Crude fiber is not included here, as the amounts were small, and its value as a source of energy is uncertain. The sum of the protein, ether extract, nitrogen-free extract, and ash is taken as 100 per cent. The lower portion of the table gives the weights of the animals. These weights are the average of the lots. Each lot contained three pigs, except Lots 38 and 39, which contained two pigs each.

TABLE IV.

Growth of Swine on Rations of Low and High Mineral Content.

Lot No.	30	31	32	33	38	39
Ration	Corn.	Corn + salt mixture.	Corn + ash-free blood protein.	Corn + ash-free blood + salt mixture.	Corn + ash-free egg white.	Corn + ash-free egg white + salt mixture.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Protein { Corn	11.20	10.88	10.00	9.55	10.00	9.56
Protein { Supplement			10.51	10.10	10.52	10.01
Fat	4.72	4.58	4.22	4.03	4.22	4.03
N-free extract	82.70	80.30	73.80	70.60	73.80	70.60
Ash	1.55	4.27	1.51	5.76	1.46	5.72
Calcium	0.017	0.66	0.17	0.99	0.17	1.01
Phosphorus	0.33	0.44	0.30	0.51	0.30	0.55
1915	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*
Initial weight { July 3	25.3	26.0	25.0	23.7		
Initial weight { Aug. 2					27.5	28.5
Final weight, Dec. 30	37.6	32.6	82.6	171.6	69.5	192.5

The average weights of the animals give the impression that Lot 32 made a greater gain than Lot 38. The individual records do not altogether support that idea, however, as one pig in Lot 32 made a much greater gain than any other receiving a similar ration.

II. Adequacy of Maize Proteins.

The rate of growth of rats receiving corn plus a salt mixture indicates in some cases an almost surprising efficiency for the corn proteins. This is the more striking if the large percentage of

zein in corn proteins is borne in mind. As has often been pointed out, this protein contains neither lysine nor tryptophane. The question is then at once presented, as to whether growth has occurred in spite of the zein, or whether zein in the ration has real value as a protein.

This problem was approached from various viewpoints. In one case the ration of corn and salt mixture was supplemented by zein itself. If the addition of this protein to maize should exert any beneficial action it would seem clear that the zein already in corn played an essential rôle in the ration of growing animals. On the other hand failure of the addition to exert any such action would not necessarily prove that all of the zein already present was without value. Again the data are not conclusive, but it does seem highly probable that the addition of zein not only had no value, but was really detrimental. Although Rat 19 made a fair degree of growth, yet on the whole the animals receiving the addition compare unfavorably with any of the others receiving a protein supplement, especially when it is noted that three of these animals are males. It seems highly probable that the addition of zein had actually lowered the value of corn.

The addition of gliadin to corn was tried because, according to Osborne and others (1915), it contains at most a very small percentage of lysine; they give 0.92 per cent as the probable value. If the addition of gliadin should cause a quickening of the growth curve, it would seem that the slow growth on corn was not due to lack of lysine in the zein. Osborne and Mendel (1911) have already demonstrated that in other respects gliadin is an adequate protein. Though it seems clear that no beneficial results could be ascribed to the gliadin, it also seems equally clear that it had no detrimental action.

Osborne and Mendel (1914) had previously demonstrated that the addition of lysine and tryptophane to zein converted it into a protein adequate for maintenance and growth; so it seemed possible that the addition of these amino-acids to corn itself might convert the latter into a more efficient diet. Accordingly two rats received corn plus 1 per cent lysine, two more received corn plus 0.5 per cent tryptophane, and two more received corn plus

1 per cent lysine and 0.5 per cent tryptophane. In none of the six cases did the addition have any evident value.[3]

The food mixture was prepared by grinding the entire maize kernel to an impalpable powder, and then intimately mixing with it the amino-acids. This dry mixture was fed to the animals without making any effort to form it into a paste, or to give it greater coherence. This procedure has one serious disadvantage in that rats are wasteful feeders, and it is difficult to obtain the exact weight of the food consumed. It seemed impossible to avoid that difficulty however, without encountering others equally serious. Observation, as well as such records as we could obtain, indicated that the rats making even moderate gains on corn alone ate relatively very large quantities of food.

In every case where corn was supplemented with a protein the amount added was such as to make the nitrogen content 3.2 per cent, or the protein content approximately 20 per cent. Thus the corn proteins were slightly less than one-half the total protein. The data are presented in Table V.

For comparison some results summarized in Table I are also presented in Table V along with other material that does not bear directly on the question of zein insufficiency. Considerable variation in the rate of growth is evidenced even among animals receiving the same food. The first two diets given, dog bread and sunflower seed, and corn and casein, are regarded as suitable for normal growth. For reasons that will be given later however, Rats 70 and 71 are considered slightly below normal in rate of growth.

It is evident that maize alone may be sufficient for maintenance and for growth, at least during a period of 7 months. In fact the rapidity of growth that may be attained on this ration is surprising. Another striking point is the very rapid rate of growth on corn when supplemented by casein.

The only notable feature concerning the feeding of egg white and dried blood is the failure of either to exert any beneficial effect. Our data on dried blood when supplied in other food mixtures are incomplete, but the records of Rats 9, 10, 11, and 12 leave

[3] These feeding trials are now being conducted under different conditions, and the results indicate that the addition of lysine and tryptophane does increase the efficiency of corn proteins.

TABLE V.

Growth (Gm.) of Rats on Corn Alone, and on Corn + Various Protein Supplements.

	Dog bread and sunflower seed.		Corn + casein.						Corn.			Corn + egg white.				Corn + dried blood.		Corn + corn gluten.		Corn + gliadin.				Corn + zein.				Corn + lysine.		Corn + tryptophane.		Corn + lysine and tryptophane.		Protein-free milk, butter, starch, and 9 per cent egg white.		Protein-free milk, butter, starch, and 15 per cent egg white.*	
Sex.	♀	♀	♂	♀	♀	♀	♂	♂	♀	♂	♀	♀	♂	♀	♀	♀	♂	♀	♀	♀	♀	♀	♂	♀	♂	♂	♀	♀	♂	♀	♂	♂	♀	♀	♂	♂
No.	77	78	70	71	73	74	75	76	60	33	34	72	67	29	30	35	36	68	69	7	8	23	24	19	20	21	22	16	15	13	14	17	18	9	10	11	12
wks.	57†	49	47	50	82	60	38	34	90	48	45	86	56	70	62	48	57	45	35	51	52	50	55	48	50	53	52	50	67	50	57	50	51	75	51	61	54
3	80	70	83	80	90	95	66	54	95	71	67	110	85	92	97	60	74	78	65	82	78	70	92	75	63	80	65	80	90	70	77	85	77	100	80	105	85
6	117	96	146	105	135	133	110	98	120	105	107	135	107	90	115	93	123	115	105	113	107	92	132	90	84	102	92	105	107	107	105	110	90	120	110	155	130
9	162	122	162	135	166	148	148	136	135	135	112	142	132	105	122	90	153	140	138	130	110	106	160	115	115	110	100	140	120	120	115	130	110	130	130	200	175
12	200	160	185	155	180	160	185	180	133	168	132	150	177	115	132	127	167	167	148	145	120	120	170	125	120			155	135	110	115						
15	210	165	195	165	186	182	228	222	140	180	135	165	180	120	130	125	172	165	150	153	135			140	122			155	140								
18	225	170	210	172	188	185	262	232	133	195	142	160	175	120	140	125	180	175	156	155	139			150	128			145	140								
21	200	185	215	175	197	190	282	242	139	210	145	172	215	125	145	130	195	180	167	175	140			156	147												
24	200	170							140	231	167	170	225	130	140	135	190	200	180	175	140			165	165												
27	215	190							145	265	190	175	255			145	195	215	205	175	150																
30	245	190							125	275	190	170				145	200	225	220																		
33									110			150																									
36									120			160																									
39									115			165																									

	gm.
* Egg white (air-dry)	92
Protein-free milk	125
Butter	144
Starch	176
Agar	25

† Initial weigh·

no doubt of the adequacy of egg white when supplied in a suitable mixture. The corn gluten[4] seemed more efficient than either the egg white or dried blood, but it is possible that this result should be confirmed by a larger number of experiments before the fact can be considered established.

A discussion of other features of the swine feeding experiment conducted at this institution is pertinent at this time. The failure of corn as a sole diet for swine may be due to the wide nutritive ratio of corn, or it may be due to the inadequacy of the proteins that are present in corn. To obtain light on that question casein[5] was added to corn in sufficient quantity to double the digestible nitrogen already present in the grain. Corn starch was then added in sufficient quantity to make the nutritive ratio of the total added material, 1: 8.8, equal to that of the corn. Three pigs received this ration, and other lots of three received corn plus various amounts of casein. The quantity of the supplement added was such that Lot 36 received a ration with a nutritive ratio of 1: 6, while this ratio for Lot 35 was 1: 3. The growth on these rations is summarized in Table VI.

TABLE VI.

A Comparison of the Value of Casein and Corn Proteins in the Ration of Growing Swine.

Lot No.	34	36	35
Ration	Nutritive ratio 1:8.8. Corn 25.00 parts. Starch 21.40 " Casein 3.00 " Salt mixture 1.24 "	Nutritive ratio 1:6. Corn 50.00 parts. Casein 2.82 " Salt mixture 1.32 "	Nutritive ratio 1:3. Corn 50.00 parts. Casein 11.68 " Salt mixture 1.54 "
	per cent	*per cent*	*per cent*
Protein (Corn)	5.64	10.29	8.77
Protein (Supplement)	4.78	4.21	14.91
Ether extract	3.02	4.97	5.92
N-free extract	82.80	76.15	65.80
Ash	3.73	4.33	4.51
	lbs.	*lbs.*	*lbs*
Initial weight, July 3, 1915	72	64	59
Final weight, Dec. 30, 1915	494	600	602

[4] Kindly furnished by the Corn Products Co. of Edgewater, N. J.

[5] Commercial buttermilk casein obtained from the Beatrice Creamery Co., Topeka, Kansas.

Reference to Table IV shows that lots receiving corn alone or corn plus a salt mixture make practically no growth. Lot 34, as is shown in Table VI, received a diet which supposedly approximates corn plus a salt mixture, but with half the digestible corn proteins replaced by casein. The procedure followed does not permit an exact comparison, but it is evident from inspection of the data that casein is far more efficient for growth than are the proteins of corn. Thus the quantity of casein substituted for corn proteins was 4.78 per cent of the ration, yet this substitution changed the ration from one barely sufficient to maintain life to a ration that sufficed for almost normal growth.

Lot 36 received a ration containing slightly less casein than did Lot 34, but nearly twice as much of the corn protein. In view of the fact that this lot gained almost 30 per cent more than Lot 34, the additional corn protein received by Lot 36 must be ranked as fairly efficient.

Another result worthy of note is the failure to secure more rapid growth by the addition of still larger amounts of casein. McCollum has indicated (1914) that up to a certain limit of protein intake, the amount retained by swine is a certain per cent of that ingested. It does not seem that his generalization would apply to these animals for the protein intake is not excessively high. The nature of the limiting factor is not clear.

Another result, of minor importance in this connection, is the evident inferiority of the blood proteins. Table IV shows that Lots 33 and 39 received rations quite similar, with the exception that in one case the protein supplement was blood protein, in another, egg white. However, the lot that received egg white made a greater gain in 5 months than the blood protein lot did in 6 months.

As has been previously mentioned, some of the rats were able to grow at a fairly rapid rate on corn and a salt mixture, but the swine seemed to utilize this ration for growth much less efficiently. Though this discrepancy might seem to indicate a fundamental difference in their metabolic processes, it is believed that such a result is to be expected. Because of the great disparity in size the energy metabolism of the rat is vastly greater than that of swine, and it ingests much more food per unit body weight. An excellent illustration of this fact is found in a summary by Arms-

by (1914) of Pettenkofer and Voit's results. For example, a dog weighing 3.10 kilos produces 2.41 times as much heat per unit body weight as does a dog weighing 30.66 kilos. On comparing rats and swine the difference is found to be much greater. According to Knapp (1908) the energy requirement per day of a 200 gm. rat is approximately 50 calories, or 250 calories per kilo. Comparable data concerning swine are not at hand, but the results obtained in the swine feeding experiment just mentioned will serve for purposes of comparison. The average weight of the pigs in Lot 36 for the last 30 days of the feeding trial was 86.4 kilos. The average daily feed consumption during that time was 1.64 kilos. The analyses of the ration are not complete enough for an exact calculation of its calorific value, but it is approximately 5,745 calories. The energy consumption per kilo of these animals, then, is 66.5 calories, or approximately one-fourth of that of a 200 gm. rat. The experimental procedures do not permit a precise comparison, but in all probability the data submitted here tend to diminish rather than exaggerate the differences. Although the energy requirement per unit body weight is much higher for the rat, the protein requirement is generally believed to be little if any higher. In other words, after each animal has satisfied its calorific needs, the rat has a much larger nitrogenous residuum with which to build new protein tissue.

III. The Presence of Growth Accessories.

A number of investigations, notably those of Osborne and Mendel, McCollum, and Funk, make it highly probable that a normal diet must contain certain "accessory" substances. These are of unknown chemical composition, and the quantity required is undoubtedly small, but nevertheless they are indispensable components of an adequate diet.

The suggestion has frequently been made that the slow rate of growth obtained when corn is the sole article of diet is due to a lack of one or more of these accessory substances. The literature on this particular point is not abundant, and is both confusing and conflicting. It might be well to note at the outset that some investigators, Abderhalden (1913) and Röhmann (1916), question the necessity of assuming that any such food

accessories, the so called vitamines, exist. Their attitude, however, is not the one commonly taken.

Most of the experiments described in this paper did not include the vitamine factor, but some of the facts presented will bear inspection from that standpoint. It is evident that, if the existence of these accessories is conceded, corn contains them in some degree. In this laboratory rats were brought to apparently normal maturity on corn and a salt mixture as the sole diet. Schaumann (1915) states that corn is "sufficient" for pigeons and rats, but insufficient for rabbits, goats, and guinea pigs. Funk (1913) has shown that the outer layer of corn contains an antineuritic substance, and a recent article by Willard (1916) states that this fact was also discovered independently by J. S. Hughes of this college. Hughes fed polished rice to pigeons until they had developed extreme cases of polyneuritis. They could scarcely move, and would make no effort to swallow. In many cases death was unquestionably only a matter of hours. If a few grains of corn were forced into the crop at this stage, the birds would make a swift recovery, and in 1 or 2 days present a wholly normal appearance. It is evident therefore that some of these accessories are present in corn.

Voegtlin (1915) in an article on the effects of a vegetable diet says comparatively little concerning accessory substances, but gives the impression that satisfactory growth could not be attained by animals fed on corn alone. Slonaker (1912) also states that a strictly vegetable diet is unsatisfactory for growing rats.

The original data submitted in this paper make it evident that rats may slowly grow to apparently normal maturity on corn alone, though some do not succeed in doing so. When corn is supplemented with casein growth occurs at a fairly rapid rate. The blood preparation seems slightly less efficient, and the egg white considerably less. Inasmuch as casein ordinarily carries large quantities of the accessories, the ineffectiveness of the egg white might seem due to a lack of those substances, and that explanation readily suggests itself. That the difference is not due to poor quality of the protein is shown by the behavior of Rats 11 and 12, Table V. These animals received egg white along with starch, butter, and protein-free milk, and grew at the maximum rate. Additional support for the views that egg

white may be lacking in accessories is found in McCollum's (1915) experience in using egg white as an adjunct to polished rice. The failure of rats to gain on polished rice has been ascribed to protein insufficiency, so McCollum supplemented the rice with ash-free egg white and a suitable salt mixture. The rats failed to grow normally on such a ration, however, thus indicating a lack of growth accessories in the egg white.

The "ash-free" casein, viewed from that standpoint, may have exerted unexpected efficiency. The only object in preparing it, however, was to obtain a product relatively free from calcium, so the material was not extracted with alcohol or ether, and no effort was made to free it of accessory substances. It is also well to note that the animals receiving such material did not grow as rapidly as those receiving the crude product. In Table V, Rats 70 and 71 received the ash-free casein, and these animals grew at a distinctly slower rate than those receiving crude casein. Corn gluten exhibits surprising value when used in this connection. As our preparation contained 4.4 per cent of ether extract, some interest is attached to Funk's (1914) statement that corn products containing considerable quantities of corn oil are richer in these unknown substances. In view of the relative superiority of casein as a supplement to corn, there is a temptation to give great weight to the vitamine factor and some effort is being made in this laboratory to investigate the theory in an experimental way. It is generally believed that an adequate diet becomes "insufficient," when heated to high temperatures, and a voluminous literature has accumulated on using such material in feeding trials. The usefulness of the procedure is open to question, as it is conceivable that factors other than "vitamines" might be subject to alteration. The method was followed here in a small way, however, for if the accessories are destroyed by heat, and rats are able to grow on heated food mixtures consisting chiefly of corn, then failure of the animals to grow on maize could not be ascribed to lack of accessories in the grain. If, on the other hand, rats fail to utilize heated foods for growth, the interpretation is not so obvious. Accordingly enough egg white to make a total of 20 per cent protein was added to corn, along with 4 per cent salt mixture, and the material was then heated for 6 hours in an autoclave at 30 pounds' pressure. Four animals

were given this ration, and their behavior on such a diet is recorded in Table VII.

TABLE VII.

Growth of Rats on a Ration Subjected to High Temperatures.

Rat No.	79	80	81	82
Initial weight.	40	43	50	42
wks.				
3	55	53	62	Dead.
6	58	57	61	
9	72	68	76	
12	70	65	87	
15	75	85	90	
18	68	87	87	
21	Dead.	88	91	
24		64	66	
27		Dead.	Dead.	

None made any noteworthy growth, and all died comparatively soon, without any evident cause, excepting that the food had been heated.

SUMMARY.

The evidence indicates that when corn is fed to rats as the sole dietary the mineral constituents are the first limiting factor, and then the protein. In the case of swine these findings are apparently reversed. Protein is here the first limiting factor, and then the mineral element. The data also indicate that the corn proteins are less efficient for growth than casein. The addition of lysine and tryptophane to maize did not increase its efficiency for growth. The addition of some of the adequate proteins (egg white) seemed of only slight benefit. In view of these facts it seems possible that one of the limiting factors in corn as a food for growing rats is one or more of the growth accessories.

Young rats on a corn diet grow more rapidly when the grain is supplemented with casein than when supplemented with egg white. This observation assumes added significance since food mixtures containing protein-free milk, butter, and egg white are more efficient for growth than mixtures of corn and egg white, even though the protein of the corn mixture furnishes a rela-

tively higher per cent of calories than does the protein of the more efficient diet.

Autoclaved corn mixtures failed to maintain body weight, and ultimate failure resulted.

Since swine grow rapidly on a mixture of corn and egg white, it is believed that corn contains sufficient of the growth accessories for normal growth in swine.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF ACETONE IN URINE.

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Frommer[1] in 1905 used salicylic aldehyde as a qualitative test for the detection of acetone in the urine. Tiemann and Kees[2] had already studied this acetone compound and Fabinyi[3] in 1900 carried the investigations still further. The alkali salt of Fabinyi's dioxydibenzene acetone

$$OH - C_6H_4 - CH = CH - CO - CH = CH - C_6H_4 - OH$$

is the compound, the red color of which was made use of by Frommer in his acetone test. Frommer's qualitative test is as follows:

"To 10 cc. of urine about 1 gm. KOH is added. Before solution has occurred 10 to 12 drops of a 10 per cent solution of salicylic aldehyde in absolute alcohol are added and the mixture warmed to about 70°C. At the zone of contact of the alkali and urine an intense purplish red ring develops in the presence of acetone."

Frommer claimed for his test the advantage over others in that it was specific for acetone as the presence of diacetic acid did not give the color reaction. Bohrisch,[4] made a critical study of the various qualitative tests and confirmed Frommer's claim of superiority in his test, and advised its general use. The specificity of the reaction suggested its use in the development of a colorimetric method for the quantitative estimation of acetone, using the distillate of the urine (which naturally includes

[1] Frommer, V., *Berl. klin. Woch.*, 1905, xlii, 1008.
[2] Tiemann, F., and Kees, A., *Ber. chem. Ges.*, 1885, xviii, 1964.
[3] Fabinyi, R., *Chem. Zentr.*, 1900, ii, 302.
[4] Bohrisch, P., *Pharm. Zentralhalle*, 1907, xlviii, 207.

the preformed acetone and that derived from the decomposition of diacetic acid), realizing the advantages such a method would possess in rapidity, specificity of color, and simplicity. The Messinger[5] iodimetric method is the one generally in use. Marriott[6] recently described a nephelometric method for the estimation of minute quantities of acetone in blood and tissues, which method in general was applied by Folin and Denis[7] to urine. The aim of the writer was to make the colorimetric method as simple and rapid as possible without sacrificing accuracy, making it adaptable also to clinical work. It is not a micro method, though applicable to minute quantities when a concentrated acetone solution obtained by a second slow distillation is used. All the above mentioned methods, excepting that of Folin and Denis, require a second time-consuming distillation to make it certain that acetone *only* is included in the result obtained.

The determination of β-oxybutyric acid by the Shaffer[8] method, in which the acid is oxidized by bichromate, is in reality an acetone determination; he used the Messinger method for determining the acetone. If glucose is present in the urine it must first be removed, since there goes into the distillate, in addition to the acetone, other volatile substances that absorb the iodine. The colorimetric method is directly applicable to the determination of acetone in the distillate; the sugar in the urine does not interfere with the reaction, since the volatile substances derived from the oxidation of the glucose *do not give the color* reaction.[9] The removal of the sugar, which Shaffer accomplished by basic lead acetate, and a second distillation, is rendered unnecessary.

By driving out all the preformed acetone before the distillation by means of an air current (Folin), it is possible to determine indirectly the amount of preformed acetone by subtracting the acetone derived from diacetic acid from the total acetone.

[5] Messinger, J., *Ber. chem. Ges.*, 1888, xxi, 3366.

[6] Marriott, W. M., *J. Biol. Chem.*, 1913, xvi, 281.

[7] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xviii, 263.

[8] Shaffer, P. A., and Marriott, W. M., *J. Biol. Chem.*, 1913, xvi, 265.

[9] 3 gm. of glucose were oxidized by potassium bichromate under conditions similar to those given in the text for the β-oxybutyric acid determination. The single distillate absorbed 12.15 cc. 0.1 N, and another sample 18.1 cc. 0.1 N iodine solution. 2 cc. of the distillate did not give any color reaction, employing the method advised in the text.

If sugar is not present in the urine the preformed acetone may be determined directly from the urine and by the subtraction of the preformed acetone from the total acetone, we may calculate the amount of diacetic acid. The presence of sugar in the urine used directly disturbs the intensity of the color reaction.

Method.

Three solutions are necessary in the estimation of the acetone: (1) A 10 per cent salicylic aldehyde solution in 95 per cent alcohol (10 gm. salicylic aldehyde made up to 100 cc. by adding 95 per cent alcohol). (2) 100 per cent KOH solution (to 100 gm. KOH, solid, are added 60 cc. distilled water). (3) The standard solution. It was found that the most convenient way to prepare the standard was by checking the concentration of the acetone (c.p.) by the Messinger method and then making a solution such that 2 cc. should contain 0.1 mg. of acetone. On allowing the standard solution to stand for a long period of time, Marriott[6] noticed the effect of polymerization on the standard solution used in his nephelometric method. It is advisable to renew the standard after 10 days, though it was found that there was no appreciable change in the color intensity after the standard had stood 2 weeks, when permanency of the color was controlled by a freshly made standard and also by a bichromate solution..

In performing the test, urine sufficient to contain 8 to 24 mg. of acetone (usually 25 to 100 cc.) are measured by a pipette into a 750 cc. flask; 5 cc. of concentrated H_2SO_4 are added and the volume is made up to about 300 cc. with distilled water. The whole is distilled for 20 minutes, using a Liebig condenser. The receiver should dip under the liquid in the receiving flask to which has previously been added 25 cc. of distilled water. The distillate is transferred to a 200 cc. normal flask.

The residue of urine from which the total acetone was obtained can be used for the determination of β-oxybutyric acid (Shaffer). Dilute the residue to about 400 cc. with distilled water; add 10 cc. concentrated H_2SO_4; distill again for 2 hours, adding drop by drop 200 cc. of a 0.5 per cent solution of potassium bichromate (for urine containing sugar use 1 per cent). The solution should be kept at moderate boiling so that at the end of 2 hours the distillate can be made up to 250 cc. in a normal flask. The acetone is determined in the distillate as described below.

2 cc. of the distillate are measured into each of two large test-tubes, for duplicate determination. 2 cc. of a 100 per cent KOH solution are added, mixed well, and then 1 cc. of 10 per cent salicylic aldehyde is added. Immediately the tubes are placed in a water bath at 45–50°C. for exactly 20 minutes to develop the color. During this time the solutions should be thoroughly mixed several times. 2 cc. of the standard solution, containing 0.1 mg. of acetone, are prepared similarly and synchronously. The tubes are removed after 20 minutes, 10 cc. of distilled water added to each tube, the contents cooled, and transferred to 25 cc. normal flasks filled to the mark with distilled water.[10] The reading should be made in a Duboscq colorimeter within 30 to 45 minutes from the time of the addition of salicylic aldehyde, setting the standard at 15 mm.

As above stated, if sugar is not present in the urine, the preformed acetone may be determined directly. The urine is made alkaline with a few drops of concentrated KOH, filtered, and the filtrate used for the direct determination of the acetone (preformed), as described above.

Since the development of the color and its intensity depend on the concentration of the reagents, special care should be taken with exact measurements. The amount of salicylic aldehyde and of KOH, and the time for the color development were tried out systematically and found to be most satisfactory when the above procedure was followed. By using pure acetone solution and urine with known amounts of acetone added, it was found in several determinations that the limit of error was ±2 per cent. The colorimetric method generally gives a lower result than the iodometric.

SUMMARY.

A colorimetric method for the quantitative determination of acetone in urine is presented, based on the red color of the alkaline salt of dioxydibenzene acetone, which was first used by Frommer as a qualitative test for acetone. The method is also recommended for clinical use on account of its simplicity, accuracy, and rapidity of estimation.

[10] In case the solution presents too intense a color, instead of using 25 cc., the volume may be made up to 50 cc. and the result multiplied by two.

THE TOXICITY OF CAROTIN.

By H. GIDEON WELLS and O. F. HEDENBURG.

(*From the Department of Pathology of the University of Chicago.*)

(Received for publication, August 23, 1916.)

In the course of an investigation of the effects of the bleaching of flour by chlorine gas, it became necessary to ascertain the possible toxicity of the pure pigment matter, both bleached and unbleached. As there could be found in the literature no report of any study of the possible physiological action of coloring matters of this class, it has seemed advisable to publish a brief account of our work, although the results are entirely negative.

It is quite certain that the coloring matter in flour is the same as that in carrots—carotin. Wesener and Teller ascribed the color in flour to carotin in 1911,[1] and suggested this in 1909. Monier-Williams[2] has advanced further proof that the coloring matter in flour is carotin, which is called "carrotene" by him.

The amount of carotin in flour is very small. Monier-Williams found in flour in parts per million as follows:

	Unbleached.	Bleached.
Flour A (photographic)	1.4	0.90
" A (colorimetric)	1.3	0.81
" B (")	2.0	1.28

The color is usually obtained from carrots, which contain it in relatively large quantities. Willstätter obtained 125 gm. from 5,000 kg. of carrots. Monier-Williams gives 2 gm. as the yield from 100 lbs. of carrots. 1.3 gm. coloring matter was obtained by us from 54 lbs. of carrots.

We obtained the coloring matter as follows: 54 lbs. of carrots yielded 21.2 kg. cleaned and trimmed material, which was ground

[1] Wesener, J. A., and Teller, G. L., *J. Ind. and Eng. Chem.*, 1911, iii, 912.

[2] Monier-Williams, G. W., *Reports, Local Gov't Board (Great Britain), Pub. Health and Med. Subjects, M Series, No. 73*, 1912.

in a meat grinder. The ground material was desiccated first by alcohol and then by distillation at 80–90° *in vacuo*. The dried mass was extracted with boiling 95 per cent alcohol. The alcohol extract yielded an extract with chloroform which was saponified with alcoholic potash. After the alcohol was removed by distillation, the residue was dissolved in water and extracted with ether. This ether extract weighed 11.2 gm. and consisted of colorless crystals mixed with carotin. The pigment was concentrated by dissolving the entire mass in carbon bisulfide and alcohol with the subsequent addition of enough water to cause the carbon bisulfide to separate, carrying with it most of the color. Several such treatments gave finally a highly colored mass that left practically nothing in the alcohol-water layer. This was now treated with alcoholic potash to remove with certainty all carbon bisulfide. After removing the alcohol the aqueous solution of the residue yielded an ether extract containing 1.3 gm. of concentrated pigment.

One-half of the pigment, 0.65 gm., was chlorinated as follows: The ether solution was shaken with a water solution of chlorine (6 cc. Cl_2 solution containing 0.01443 gm. Cl_2 per cc.). 0.65 gm. carotin, $C_{40}H_{56}$, requires by theory 0.0861 gm. Cl_2 to form $C_{40}H_{56}Cl_2$, and our material used 0.08658 gm. Cl_2. The color of the ether solution decreased and the Cl_2 odor vanished. The chlorinated product weighed about 0.09 gm. more than the initial amount.

In subsequent experiments we obtained but 1.2 gm. pigment from 200 lbs. of carrots, which vary greatly in pigmentation at different seasons.

This purified pigment was used in the following experiments. As it is soluble only in fat solvents, which are all more or less toxic, it was used dissolved in sterile olive oil. This would seem to be a suitable solvent, however, as shown by the efficiency of phlorhizin dissolved in oil when used in metabolism studies. Because of the extremely minute quantity of coloring matter contained in flour, it was not feasible to conduct experiments with carotin from flour, as only 1 gm. of color is contained in 1,000 kg. of flour, and in view of the demonstrated identity of the coloring matter of flour and carotin from carrots the investigation of the latter seemed sufficient.

Two pairs of male guinea pigs were given intraperitoneal injections of carotin dissolved in olive oil and sterilized by brief heating on the water bath.

N 1.	Wt. 339 gm.,	given	3	cc.	oil	with	0.100 gm.	natural carotin.
P 1.	Wt. 316 "	"	3	"	"	"	0.100 "	chlorinated carotin.
N 2.	Wt. 265 "	"	5	"	"	"	0.200 "	natural carotin.
P 2.	Wt. 245 "	"	5	"	"	"	0.200 "	chlorinated carotin.

These animals were kept under close observation for 12 hours, the temperature being taken every 2 hours. At the end of 4 days, during which they were frequently examined, they were killed with illuminating gas. Practically no effects were observed. All showed a rise of 1–2°F., 2 to 6 hours after the injection, but so did control pigs given olive oil alone. They all remained quiet but alert, and ate as well as the control pigs. The urine at no time showed albumin or sugar, but was of a deep reddish brown color. The autopsies showed no changes except the presence of a considerable amount of fatty yellow material in the peritoneum, but much less than the amount injected. There were no signs of inflammation or other local changes. All the viscera seemed normal in both gross and microscopic examination.

Evidently carotin possesses no considerable toxicity, if any, and chlorination does not make it demonstrably toxic. The quantities of carotin used in these experiments are enormous in comparison to the amounts that could be obtained from any food, even carrots. The larger dose used, 0.2 gm., is as much as is contained in 200 kg. of flour, or as lutein in 4,000 cows' ovaries (Escher[3]).

Local or Cellular Toxicity.

Although the absence of either symptoms or anatomic evidence of irritation after intraperitoneal injections of carotin indicated that it can have little or no local effect, this was determined more definitely by a series of intradermic tests. This method, devised and used in this laboratory by Dr. Corper[4] for the study of local toxicity, has been found very useful for this purpose. The principle and method are similar to those used in the intradermic

[3] Escher, H. H., *Z. Physiol. Chem.*, 1913, lxxxiii, 198.

[4] Corper, H. J., *J. Biol. Chem.*, 1915, xx, p. xxi.

injection of tuberculin or diphtheria toxin. As the two preparations to be compared are injected side by side in the same animal, no individual peculiarities of the animal enter as a source of confusion or error.

The carotin, dissolved in varying concentration in olive oil, was injected in parallel sites intradermically in 0.2 cc. quantities into two guinea pigs. The effects were the same in both.

Carotin dissolved in olive oil to the strength of 10 per cent seems to have a very slight local toxicity, as shown by the development of a little edema and swelling. At 1 per cent the results are very little different from those with olive oil alone. These results are not appreciably different with chlorinated and natural carotin. Olive oil containing very small amounts of carotin (0.1 per cent) seems to cause, if anything, slightly less protracted swelling than olive oil alone; possibly a slight irritant effect from carotin hastens absorption.

These experiments indicate that even in relatively very large doses carotin, whether in its natural state or saturated with chlorine, is almost entirely devoid of toxicity. Such large amounts as 20 mg. injected intradermically cause only a local edema and inflammation, but no necrosis.

The extensive studies of Palmer and Eckles[5] indicate that carotin is almost universally distributed throughout all animal bodies, coming chiefly, if not solely, from the food. Our few experiments seem to be sufficient to warrant the assumption that any such quantities as can ever accumulate in the tissues have no harmful effects.

[5] Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191.

CLINICAL CALORIMETRY.

XVIII. THE NUMBER OF PLACES OF SIGNIFICANT FIGURES IN THE DATA OF METABOLISM EXPERIMENTS.

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In the publications from this laboratory and many others results have often been expressed with more decimal places than are justified by the accuracy of the methods or the significance of the findings. This involves a waste of time and printers' ink and gives the reader a false impression of the work. It is extremely desirable to get along with the smallest number of digits that will do justice to the experiment and yet allow other investigators to recalculate the work.

In the publication of data three factors must be considered: (1) the accuracy of the analytical results; (2) the possible errors in the methods of calculation; and (3) the significance of the findings. It may be well to consider them in this order.

There is great variation in the accuracy of the different methods of analysis used in metabolism experiments. An attempt is usually made to keep all possible errors within the limits of 1 per cent, but few investigators believe that they can do this in all their work. There are some methods in which the error may be distinctly less than 1 per cent but they are usually affected by some other factor in which the error is much greater.

Holman,[1,2] in his standard work on computations, gives, among others, the following principles and rules which are well worth repeating:

"Retain everywhere enough places to correspond to two unreliable places in the final result; the direct object of this is to keep the first place

[1] Holman, S. W., Computation Rules and Logarithms, New York, 1915.

[2] Holman, Precision of Measurements, New York, 2nd edition, 1904.

of unreliable figures in the final result substantially free from the accumulated rejection errors.

Exceptions: A final result is seldom stated to more than one uncertain place unless the uncertainty of that place is small (say plus or minus four or less).

In multiplication or division, the percentage accuracy of the product or quotient cannot exceed that of the factor whose percentage accuracy is least.

In addition or subtraction, the result cannot be accurate beyond the first decimal place which is inaccurate in any component.

In casting off places of figures, increase by 1 the last figure retained when the first left-hand rejected figure is 5 or greater; otherwise leave it unchanged.

A mean or average should always be carried to two unreliable figures.

A mean is more reliable than the single observation from which it is computed (in proportion to $\sqrt{n}$, the square root of the number of observations)." Undue importance should not be attached to this numerical relation when the number of observations is very small, as for instance when not exceeding 5 or 10.

"In direct multiplication or division, retain in every factor, product, and quotient throughout the entire process, and in final results, for an accuracy of about one per cent, or worse, four places of significant figures; one-tenth per cent, or worse, five places of significant figures; and so on.

If the multiplication or division is performed by means of logarithms, the mantissa should contain as many places as are required by the foregoing rules for the direct process; *i.e.*, for about one per cent, or worse, use four place tables; one-tenth per cent, or worse, use five place tables.

In addition or subtraction . . . . , for an accuracy of about 1 per cent, or worse, carry the result to four places of significant figures, etc.

If a single observation deviates widely from the others of the series and no source of error can be discovered it should be published but not used in determining the mean except as follows.[3] "Take the mean and the (average deviation) *a.d.* from this mean of the observations omitting the doubtful one. Find the deviation (*d.*) of that one from the mean. Then reject the observation if $d. > 4\ a.d.$ . . . . Something must also be left to the judgment of the observer as to the propriety of making a rejection."

As Holman[3] goes on to say: "There is a tendency, especially among inexperienced observers, to become biassed by the first one or two readings of a series, and to reject, without recording it, any later one which does not closely accord with these, tacitly assuming it to be faulty. This is an essentially vicious practice which cannot be too carefully avoided. Other things being equal the later observations are entitled to greater rather than less weight than the earlier ones, and no result should be rejected without sufficient warrant.

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[3] Holman, Precision of Measurements, New York, 2nd edition, 1904, 30.

The rules[4] are so framed that, barring mistakes, the greatest possible computation error entering into the result of any ordinary computation (*e.g.*, one involving a total of not much exceeding twenty component numbers, steps, or operations, where a rejection error may occur) shall not be sensible compared with the errors of the measurements or data, or shall not sensibly affect the accuracy of the results. They are, therefore, *safe* rules in the worst possible cases. But in order to be so they are necessarily more than sufficiently stringent for some classes of comparatively rough work, where the *infrequent* undetected entrance of a computation error two to four times as large as the experimental error would be permissible. For such work, one less place of figures may be used, but when the rules are thus relaxed the possible consequence should be borne in mind, and special scrutiny applied to the various stages of the computation, special attention being directed to quantities beginning with 1 or 2."

The maximum rejection error possible in any given equation can be readily calculated from the formula $\frac{5n}{10^r}$, n representing the number of factors, quotients, and products at which rejections are made and r representing the last place retained. 5 is, of course, the maximum rejection error in any one number and it is conceivable that it might be rejected in the same direction in every case. The greatest percentage error will occur if every factor begins with the digits 10. In order to make $\frac{5n}{10^r} = \frac{1}{100}$ or 1 per cent we can have the following combinations: $r = 3$ and $n = 2$, or $r = 4$ and $n = 20$. Therefore, if we use only two factors we need retain only three places to have the maximum error 1 per cent. If we have 20 factors, quotients, etc., we must retain four places. It is extremely improbable that the figure rejected would be $+5$ or -5 in every case and the natural tendency would be for the rejections to neutralize each other. It is also improbable that all the factors would begin with 1. If, for instance, any factor began with 5, the above percentage error would be divided by 5.

In some of the calorimeter calculations there are as many as fifteen or twenty multiplications, divisions, etc., and it is obvious that for such work four places should be retained until the end. On the other hand, analyses of food, urine, etc., involve simple

[4] Holman, Computation Rules and Logarithms, New York, 1915, p. xliii.

calculations with only three or four factors, some of them being constants. For such cases the average error would be small and the maximum error infrequent if the fourth place of each titration or weighing were rejected at the start. If n were 4 the maximum error would be $\frac{5+5+5+5}{10.0 \times 10.0 \times 10.0} = \frac{20}{1000}$. The probable error would be more like the following example: $\frac{5-3+4-1}{15.0 \times 84.0 \times 32.0} = \frac{5}{4032}$. Even if the maximum error did occur it would be diminished in the averages. The safest rule, however, is to retain through the calculations four places wherever possible. There is no need of retaining five places in any metabolism work except, perhaps, in a few of the calorimeter calculations where we are dealing with small differences between large numbers. The use of five place logarithm tables can be almost entirely abandoned if the interpolations be carefully made in four place tables.

If slide rules be used, it is usually considered that a 20 inch rule corresponds to a four place logarithm table.

When we come to the publication of the results the matter is entirely different. The calculations have been made and the dangers from rejection errors have been passed. Subsequent investigators may recalculate the findings but such recalculations are seldom complicated and do not involve many divisions or multiplications. After all, a man who recalculates the work from another laboratory does not expect the error to be less than 1 per cent.

Even the man who publishes does not often claim greater accuracy than this. Were the analytical results accurate within 0.1 per cent, there would be no significance in such accuracy. It is hard to conceive of a 1 per cent difference causing any change in the interpretation of any one metabolism experiment. A 1 per cent difference would be of significance only in the mean of a large number of experiments. If final results are expressed in three places they will always be within the 1 per cent limit and, except for numbers beginning with 1, they will be far within this limit.

Each investigator will probably decide for himself the number of significant figures that he will publish. It is the purpose of

the present paper to standardize the number of places used in the publications from the Russell Sage Institute of Pathology. If the number of places is smaller than heretofore used it must not be felt that the accuracy of the work has been impaired or has been considered inferior to that of other laboratories. Analyses of food, urine, and feces are always made in duplicate and standard methods used. The bomb calorimeter is of the best type made. The respiration calorimeter is probably the most accurate machine of its kind ever constructed. In the metabolism ward the collection of specimens is all that could be desired except in the case of very stupid patients. An endeavor is made to keep the administration of foods to the patients as accurate as the analytical methods. Only foods which vary but slightly in composition are used and these are weighed before cooking, samples being taken from time to time for analysis. The most uncertain quantity is the patient himself or rather the physiological law which is being investigated. Nature does not work within 1 per cent limits, especially in disease.

A table is given showing the number of places to which results will be expressed in the publications from this Institute. In most cases three significant figures will be used; in some where the analysis is not quite so exact only two will be given. In some of the analyses of food, blood, and feces, either two or three figures will be used, depending on the amounts present and the methods of analysis. It must not be felt that this table is intended to disparage the accuracy of any particular method of analysis. The rejections of the third and fourth places have been made chiefly because they lack significance. For example, we could determine the total nitrogen of the urine and feces to 0.01 gm. but if we did not analyze the sweat, the first decimal place in the total excreta nitrogen might show a considerable error. Again, the third decimal place in the respiratory quotient is variable and of no significance except in alcohol checks and in severe diabetes where it is of considerable interest and of approximate accuracy in many cases.

SUMMARY AND CONCLUSIONS.

In metabolism work the analytical error is seldom much less than 1 per cent. A variation of 1 per cent in the result of an experiment would not change its significance or affect its interpretation. For these reasons, it seems unnecessary to publish more than three significant figures in the tables of data and in some cases it is not worth while to publish more than two. In order to avoid the accumulated rejection error it is advisable to retain four figures wherever possible in the calculations and reject the fourth digit only in the final result.

TABLE I.

The Number of Places of Significant Figures Adopted as Standards in the Publication of Metabolism Data.

	No. of significant figures.	Examples.		Unit.
Urine.				
Volume	3	1,560.	654.	Cc.
Total nitrogen	3	12.4	7.45	Gm.
Glucose	3	28.2	9.84	
Ammonia N	3	1.24	0.777	
Urea N	3	11.3		
Creatine N	3	0.013		
Creatinine N	3	0.658		
Uric acid N	3	0.451		
Purine base N	2	0.031		
Amino-acid N	3	0.681		
Sodium chloride	3	14.5		
Total sulfur	3	0.902		
Total phosphorus	3	1.46		
Acetone	3	5.23	0.999	
Diacetic acid	3	4.43	0.851	
β-Oxybutyric	3	24.5	2.45	
Acidity 0.1 N	3	246.		Cc.
D : N	3	3.65		
Feces.				
Nitrogen	3	1.34	0.971	Gm.
Fat	3	12.5	6.34	
Carbohydrate	2	1.2		
Ash	2–3	0.98	2.45	
Moisture	3	56.4		

TABLE 1—*Continued.*

	No. of significant figures.	Examples.		Unit.
Food (analyses).				
Heat of combustion	3	3.24		Cal.
N analysis	3	10.1		Gm.
Protein (N × 6.25)*	3	63.1		
Ether extract	3	17.5		
Phosphorus	3	1.52		
Carbohydrate	3	83.4		
Sodium chloride	3	1.33		
Moisture	3	54.5		
Food (given in ward).				
Calories	3	2,340.		Cal.
N	3	14.5		Gm.
Fat	2	24.		
Carbohydrate	2	250.		
Sodium chloride	2	16.		
Water	2	2,500.0		
Blood.				Mg.
Urea N	2–3	102.	23.	
Non-protein N	2–3	201.	52.	
Glucose	2–3	132.	89.	
Creatinine	2	13.	1.3	
Uric acid	2	11.	2.1	
Chlorides	3	592.		
Fat	2	230.	140.	
CO_2 combining capacity	2	69.		Volume per cent.
Body measurements.				
Weight	3	74.3		Kg.
Height	3	178.		Cm.
Surface area	3	1.85		Sq. m.
Temperature	3	37.2		°C.
Respiratory metabolism.				
CO_2	3	24.6		Gm.
O_2	3	23.4		"
H_2O	3	34.7		"
Calories, direct	3	65.9		Cal.
Calories, indirect	3	66.3		"
Respiratory quotient	2	.82		
R. Q., severe diabetes	3	.697		
Per cent calories from protein fat and carbohydrate	2	17.		Per cent.

*This factor is for general use. For special cases it is more desirable to separate the protein and weigh it as such, or in cases where the nature of the protein is known to apply the proper conversion factor.

THE PERMEABILITY OF LIVING CELLS TO ACIDS AND ALKALIES.

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(Received for publication, August 10, 1916.)

Recent investigation has rendered it increasingly clear that one of the most powerful means of influencing metabolism is by changing the reaction of the protoplasm. In view of this, the penetration of acid and alkali into the cell has taken on additional interest.

Some of the most important contributions to this subject may be briefly mentioned. De Vries[1] found that the red sap of the cells of the red beet turns brown when placed in NH_4OH, but that on washing in water the red color again returns. Pfeffer[2] showed that the red sap of *Pulmonaria* petals, of the stamen hairs of *Tradescantia*, and of sections of beet root first becomes blue and then greenish in dilute NH_4OH, KOH, or K_2CO_3. Pfeffer[3] mentions that as a rule the original color returns when the tissue is well washed with water, while if the reagents are sufficiently dilute and the action not too prolonged, the cells remain living for a time.

Plant cells have not been utilized in recent investigations on the permeability of protoplasm to acids and alkalies, chiefly[4] because the blue anthocyan pigments of petals were considered not to be sensitive enough to weak acids (such as acetic) and also because of the fact that many plant cells are cuticularized and are therefore not readily wetted or penetrated by the solutions.

Bethe[5] found that neutral red could be introduced into cells as an indicator in the study of permeability. Warburg[6] in his work on the permeability of sea urchin eggs to alkalies, made use of this dye in staining the

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[1] De Vries, H., *Arch. Néerland. sc. exact. et nat.*, 1871, vi, 124.

[2] Pfeffer, W., Osmotische Untersuchungen, Leipsic, 1877, 140. Ruhland, W. (*Jahrb. wiss. Bot.*, 1909, xlvi, 1), has confirmed these observations.

[3] Pfeffer, The Physiology of Plants, Oxford, 2nd edition, 1903–06, i, 92.

[4] Harvey, E. N., *Science*, 1914, xxxix, 947.

[5] Bethe, A., *Arch. ges. Physiol.*, 1909, cxxvii, 219.

[6] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.

cells. Harvey,[7] in his studies on the penetration of cells to alkalies found it difficult to secure animals whose cells contain a pigment that shows a marked color change on addition of alkali. In the absence of a suitable pigment he introduced neutral red into the cells, a method which has been criticized recently[8] but which Harvey[9] has shown to be unaffected by proteins, lecithin, etc., in respect to sensitivity to different alkalies. It was found[9] that living cells would not stand staining with indicators of acids (as had been done previously for alkalies), so that organisms with natural pigment had to be found. Though many pigmented animals are known to occur, those containing suitable indicators are quite rare.

While on the Barrier Reef expedition,[9] Harvey obtained *Stichopus ananas*, the "prickly fish," which contains a natural indicator. The pigmented testis of this form served as material for his study of the speed of acid penetration of protoplasm. Crozier,[10] at the Bermuda Biological Station, found *Chromodoris zebra*, a nudibranch, containing a suitable indicator, which he utilized in studying the penetration of acids. Further investigations at Bermuda have enabled Crozier[11] to describe the color changes of indicators derived from four pigmented animals, showing that natural indicators in animals are not quite so rare as had previously been supposed. As investigators have found it necessary to go to a considerable distance to secure naturally pigmented animals suitable for such studies, it seems very desirable to ascertain whether plant tissues suitable for such studies are not readily available.

The writer has found that many blue petals are admirably adapted to the study of the penetration of acids and that many red petals and other parts are well suited to the study of the penetration of alkalies. In the course of these experiments the writer has made extensive use of the following: 1. Petals of *Browallia speciosa*. 2. Petals of *Pelargonium*. 3. Perianth of "Queen of the blue" hyacinth. 4. Root of red radish.

It is evident that in such investigations it is important to compare the rate of penetration of (1) acids and alkalies of the same normal concentration as found by ordinary titration, and (2) acids and alkalies of the same hydrogen ion concentration as found by means of the gas chain. Accordingly, a series of 0.01 N acids and alkalies were prepared by ordinary titration methods. A similar series of acids of 0.01 N hydrogen ion con-

[7] Harvey, *J. Exp. Zool.*, 1911, x, 507.
[8] McClendon, J. F., and Mitchell, P. H., *J. Biol. Chem.*, 1911–12, x, 459.
[9] Harvey, *Am. J. Physiol.*, 1912–13, xxxi, 335.
[10] Crozier, W. J., *Science*, 1915, xlii, 735.
[11] Crozier, *J. Biol. Chem.*, 1916, xxiv, 268, 443.

centration and alkalies of 0.01 N hydroxyl ion concentration was made up by means of the gas chain. Approximately 50 cc. of each solution were run into each separate tumbler. The tumblers were ordinarily kept covered with glass plates. Blocks of rubber that had been boiled in water for several days were slit so that the plant material could be firmly held in the slit. After the insertion of the material into the rubber, the edge of the tissue was cut across smoothly by means of a sharp razor. This razor cut was made to allow the acid to penetrate through the uncuticularized walls of the cells, since it was found that penetration through intact cuticle is excessively slow. The cutting does not appear to injure in any way the cells in the neighborhood of the cut or to affect the color changes which were used as a criterion of penetration. The rubber cubes, below the surface of the solution, hold the material and should any air bubbles be attached to the tissue, they are readily removed with the help of a glass rod.

The criterion of penetration was the first appearance of a very narrow, just distinguishable, band along the cut edge which was observed with the use of the daylight lamp.[12] If the cells are washed in distilled water when the first change of color appears, the normal color reappears. The primary object of the experiments has been to ascertain whether or not the same relative order of penetration found with animal tissues obtained also in the case of plant tissue. The experiments were run for as short a time as possible, usually less than $\frac{1}{2}$ hour and seldom over 1 hour.

When a very weak acid is penetrating the cells very slowly, the indicator may be broken down before sufficient acid has entered the cell in order to cause a change of color in the indicator. When such cases occur they can be readily recognized by making an extract and then adding the indicator at various rates of speed to the acid in a test-tube. Short exposure of the plant material to the solutions decreases the possibility of errors due to pathological conditions.

In the following tables the acids are arranged in order of penetration, the most rapidly penetrating being placed first.

[12] Crozier, *Science*, 1915, xlii, 764.

Each table gives the results of several (five to ten) series of experiments.

TABLE I.

Browallia Petals.

0.01 N total alkali (titration).	0.01 N total acid (titration).
Ammonium hydroxide.	Salicylic.
Sodium "	Benzoic.
Potassium "	Trichloroacetic.
	Formic.
	Hydrochloric.
	Nitric.
	Sulfuric.
	Phosphoric.
	Oxalic.
	Tartaric.
	Lactic. } Very slow.
	Citric. }
	Acetic. Extremely slow.

TABLE II.

Hyacinth Perianth.

0.01 N total acid (titration).

Benzoic.
Salicylic.
Trichloroacetic.
Hydrochloric.
Nitric.
Sulfuric.
Oxalic.
Formic.
Phosphoric.
Tartaric.
Lactic.
Citric. } Very slow.
Butyric. }
Acetic. }

TABLE III.

Pelargonium Petals.

0.01 N total alkali (titration).

Ammonium hydroxide.
Sodium hydroxide; potassium hydroxide.

Table I shows that the results with *Browallia* petals coincide with the results obtained with 0.01 N acids by Harvey and Crozier, working with animal tissues, except that in Harvey's experiments lactic and oxalic acids penetrated more rapidly than phosphoric. This difference is probably of little significance, since oxalic acid penetrates more rapidly than phosphoric into the perianth of the hyacinth (Table II).

Test-tube experiments with the natural indicators showed that on adding 0.01 N acetic or butyric acids the indicators broke down and changed color in such a way that they could not furnish a very satisfactory criterion of penetration. Acids which affect the indicators in this manner probably enter the cell slowly as a rule and may cooperate with other factors to decompose (or isomerize) the indicator.

The relative penetration of alkalies shown in Tables I and III agrees with the results of Harvey.

It is evident that as the hydrogen ion is the agent which causes the change of color in the cell (and doubtless is primarily responsible for the effect of acids on metabolism), we should test the penetration of the hydrogen ion in solutions which have the same hydrogen ion concentration. This, as far as the writer is aware, has never been done. Accordingly, a series of acids having a hydrogen ion concentration of 0.01 N and of alkalies having a hydroxyl ion concentration of 0.01 N, was prepared by the use of the gas chain. The results are shown in Tables IV, V, and VI.

TABLE IV.

Browallia Petals.

0.01 N hydrogen ion concentration (gas chain).	
Acetic.	
Formic.	
Lactic.	
Trichloroacetic.	Approximately all the same.
Sulfuric.	
Oxalic.	
Nitric.	
Hydrochloric.	
Tartaric.	
Phosphoric.	
Citric.	

TABLE V.

Hyacinth Perianth.

0.01 N hydrogen ion concentration (gas chain).		0.01 N hydroxyl ion concentration (gas chain).
Acetic.		Ammonium hydroxide.
Formic.		Sodium "
Lactic.		
Oxalic.	Approximately all the same.	
Tartaric.		
Phosphoric.		
Hydrochloric.		
Citric.		
Sulfuric.		
Nitric.		
Trichloroacetic.		

When we compare the results obtained by making 0.01 N solutions by titration (Tables I, II, and III) with the results obtained by making solutions of 0.01 N hydrogen ion concentration by the use of the gas chain (Tables IV and V), we see striking differences. The most interesting is the fact that acetic jumps from the bottom to the top of the list; a similar change is seen in the position of lactic acid while a much smaller change occurs in the position of formic acid.

This is exactly what would be expected if the change in the color of the cell depends primarily on the penetration of the hydrogen ion. For when the solutions are made up, as in Tables IV and V, the hydrogen ion concentration of acetic acid is increased (as compared with the solutions in Tables I and II) more than the hydrogen ion concentration of lactic or formic, and consequently it ought to change its position on the list more than either lactic or formic.

Formic acid (ionization constant 0.0214) dissociates more than lactic acid (ionization constant 0.0138) and the latter more than acetic acid (ionization constant 0.00187), so that in a 0.01 N solution of formic acid (made by titration) there is a much higher concentration of hydrogen ions than in 0.01 N lactic acid and 0.01 N lactic acid has a greater hydrogen ion concentration than 0.01 N acetic acid. Hence we should expect 0.01 N formic to show greater penetrating power than 0.01 N lactic, and 0.01 N lactic

greater than 0.01 N acetic, when the solutions are made up by titration as in Tables I, II, and III.

If the concentration of the hydrogen ion were the only factor governing penetration, we should expect all the acids in Tables IV and V to penetrate at the same rate. As a matter of fact, acetic penetrates more rapidly than formic and the latter more rapidly than lactic while the rest go in more slowly than lactic. The reason for these differences must be left to further investigation.

Similar considerations apply to the penetration of alkalies. If the hydroxyl ion concentration were the only factor governing the penetration of alkalies, we should expect both the alkalies in Table V to penetrate at the same rate, but we find that ammonium hydroxide penetrates more rapidly than sodium hydroxide.

An apparent basis for comparing the rate of penetration of hydrogen and hydroxyl ions is afforded by Table VI. The material (root of red radish) shows a change whether placed in acids or in alkalies, and hence we may roughly compare the penetration of the two. This, however, is of doubtful significance because we are unable to say whether the changes in color produced by the acid and alkali correspond to equal changes in hydrogen ion concentration.

TABLE VI.

Root of Red Radish.

Concentrations expressed as normal concentrations of hydrogen or hydroxyl ion (gas chain).

Series A.	N.
Acetic	0.0022
Hydrochloric	0.003
Sodium hydroxide	0.002
Series B.	
Acetic	0.0012
Hydrochloric	0.0013
Sodium hydroxide	0.001
Series C.	
Acetic	0.00045
Hydrochloric	0.00047
Sodium hydroxide	0.0008

SUMMARY.

1. Plant tissues containing natural indicators are admirably adapted for studying the penetration of acids and alkalies.

2. The relative rate of penetration of the hydrogen ion in 0.01 N solutions (made by titration) of acids and alkalies is practically the same in the plants studied as that found by Harvey and Crozier for animals.

3. Very different results are obtained when the solutions are made up with a hydrogen ion concentration of 0.01 N (by the use of the gas chain). We then find, for example, that acetic acid is at the top instead of at the bottom of the series (some other acids also change their position). This may be explained in part by the dissociation of the acids concerned.

THE ACIDITY OF PLANT CELLS AS SHOWN BY NATURAL INDICATORS.

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(Received for publication, August 14, 1916.)

The importance of ascertaining the actual reaction of protoplasm has become more and more evident with the progress of investigation. In view of this, the writer has endeavored to discover to what extent natural indicators, found in living cells, can be utilized to determine their reaction.

The value of this method is due to the fact that indicators show the actual rather than the total acidity, and it is the actual acidity which is of chief importance for life processes. The writer has found[1] that in plant cells there is no constant relation between the two.

The total acidity includes both the undissociated and the dissociated hydrogen ions, while the actual acidity depends only on the latter and is conveniently designated by the $P_H{}^+$ numbers of Sörensen, which are the negative common logarithms of the numbers expressing hydrogen ion concentration. Thus if the hydrogen ion concentration is $1 \times 10^{-7.5}$ N, the $P_H{}^+$ number is 7.5.

Before describing the results it may be of interest to mention briefly some previous contributions to the subject.

It is stated by Pfeffer[2] that the red color of rose petals, beet roots, etc., indicates acidity, while the blue coloration of the bluebell and hyacinth is taken as an indication that these cells are neutral or slightly alkaline. He also states that treatment of the cells with acid or alkali may change the reaction without at once killing the cells and that upon thorough washing, the color of the normal reaction returns. Flowers of *Pulmonaria* have been observed to change from red to blue as they grow older, indicat-

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[1] Haas, A. R., *Bot. Gaz.* (in press).

[2] Pfeffer, W., Osmotische Untersuchungen, 1877, 140; *Untersuch. Bot Inst. Tübigen*, 1886, ii, 293.

ing a change in the reaction. Kraemer[3] has succeeded in modifying the color of flowers by adding reagents to various kinds of soil. (When cut flowers and plants were supplied with dyes or color substances, only a few were carried as high as the flower, indicating that only certain substances could be taken up by the plant and exert an influence on the coloring matter in the flower.) Blue chicory flowers have been observed by Kastle and Haden[4] to change from blue to white and finally to brown, with practically complete destruction of the flower pigment, due in part to variations in acidity and in part to the oxidase action which completely oxidizes and destroys the flower pigment. The red, blue, and colorless solutions of the blue chicory flower pigment were found to be acid to phenolphthalein and even the yellowish green solutions did not always give an alkaline reaction with phenolphthalein. The color change of phenolphthalein is, according to Sörensen's table, colorless to red, $P_H + 8.3$ to $P_H + 10.0$, which indicates that a solution may still be colorless to phenolphthalein and be alkaline.

The experiments of the writer were carried on with a variety of anthocyan pigments,[5] especially those of petals. Buffer solutions from $P_H + 1$ to $P_H + 13$ were made up by means of the gas chain, so that the hydrogen ion concentration of each solution was accurately known. Aqueous or alcoholic extracts of the pigments were made by rapidly macerating the petals in a mortar with the solvent. The activity of enzymes in destroying the pigments required that in many cases the buffers be handled rapidly. The extracts were made as concentrated as possible and to each buffer solution the same number of drops of the extract of pigment was added. The resulting colors are given in the following tables. Rapid decolorization of the pigment is indicated by a vertical line. Petals are used unless otherwise stated.

[3] Kraemer, H., *Science*, 1906, xxiii, 699; 1909, xxix, 828.

[4] Kastle, J. H., and Haden, R. L., *Am. Chem. J.*, 1911, xlvi, 315.

[5] It is chiefly to Willstätter and his students that we owe our knowledge of the nature of anthocyan. Willstätter, R., *Ber. chem. Ges.*, 1915, xlvii, 2831; *Ann. Chem.*, 1914, cdviii, 1; *Sitzungsber. Akad. Wiss. Berlin*, 1914, xii, 402; *Ann. Chem.*, 1913, cdi, 189. See also Crocker, W., *Bot. Gaz.*, 1916, lxi, 349, and Wheldale, M., *J. Genetics*, 1914, iv, 113; 1910–11, i, 134; *Proc. Roy. Soc., Series B.*, 1909, lxxxi, 44; *Proc. Cambridge Phil. Soc.*, 1909, xv.

TABLE I.

Flowers.

P_H^+	Sweet pea. Lavender. 95 per cent alcohol extract.	Pansy. Blue. 95 per cent alcohol extract.	*Primula obconica.* Pink (flowers turn blue, then white in fading). Water extract.	"Queen of the blue" hyacinth. Blue. Water extract same as alcohol extract.	Sweet pea. Pink. Water extract.	Pansy. Blue. Water extract.
1	Pink.	Rose.	Rose.	Pink.	Pink.	Rose.
2	"	"	"	"	"	"
3	Lavender.	Pink.	Pink.	"	"	"
4	Pale lavender.	Violet.	"	Pink-blue.	"	"
5	"	Blue.	Red-violet.	Blue.	"	Blue.
6	"	Blue-green.	Violet.	"	Pink-violet.	Blue-green.
7	Green.	"	Blue.	Blue-green.	Blue.	"
8	"	Green.	Green.	Green.	Blue-green.	"
9	"	Yellow.	"	"	Green.	Green.
10	"		Green to yellow.	"	"	"
11	"				"	"
12					Yellow.	Yellow.

TABLE II.

P_H^+	*Cichorium intybus*. Blue. Water extract.	Violet. Blue. Water extract.	*Scilla*. Blue. 50 per cent alcohol extract.	Hyacinth. Red. 95 per cent alcohol extract.	*Primula chinensis*. Red. 95 per cent alcohol extract.	*Primula chinensis*. After red petals had changed to blue. 95 per cent alcohol extract.
1	Pink.	Pink.	Pink.	Red-orange.	Not determined.	
2	"	"	"	"	"	
3	Blue.	"	"	Light orange.	"	
4	"	"	Pink to violet.	Pink.	"	
5	"	Violet.	"	"	Pink.	P_H^+ 6.23 violet.
6	"	Blue.	Blue.	"	"	P_H^+ 6.5 blue by reflected light.
7	Violet.	"	"	Violet.	Blue.	P_H^+ 6.97 brown-green.
8	Blue-green.	Blue-green.	Green.	Green.	Blue-green.	P_H^+ 7.38 green.
9	Green.	Green.	"	"	Green.	P_H^+ 8.04 green.
10	"		"			

TABLE III.

P_H+	*Pelargonium.* "Paul Crample." Pink. 95 per cent alcohol.	Hyacinth. Dark blue. 95 per cent alcohol.	*Browallia speciosa.* Blue. 95 per cent alcohol.	Blue violet. Extracted with 95 per cent alcohol; filtered; evaporated to dryness; dissolved in water as indicator.		Blue pansy. Water extract of indicator obtained in same way as for blue violet.	
					*		†
1	Orange-yellow.	Red.	Red.	Red.	Red.	Red.	Red.
2	"	"	"	Rose.	Rose.	Rose.	Rose.
3	Pink.	Pink.	Pink.	Pink.	Pink.	Pink.	Pink.
4	"	"	"	Pink-violet.	Pink-violet.	Violet.	Violet.
5	"	Blue.	"	Blue-violet.	Blue-violet.	Blue-violet.	Faint blue.
6	"	"	Blue.	Blue-green.	Green.	Purple.	Green.
7	"	Blue-green.	"	"	"	Blue.	"
8	Blue.	Green.	Blue-green.	Green.	"	Blue-green.	"
9	Violet.	"	Green.	Green-yellow.	Yellow.	Green.	
10	"		"	"	"	Yellow.	
11	Green.		"	Yellow.	"		
12	"		Yellow.	"	"		

*After standing in buffer solutions 15 hours.

†After standing in buffer solutions several hours.

TABLE IV.

P_H^+	Blue violet.*	Red cabbage leaves. Purple. Water extract.	Cranberry juice on paper. Red. When dry, touched with drop of each buffer solution.	Cranberry peelings boiled with water to extract the pigment. Red.	Radish root. Red. 95 per cent alcohol extract.	Red beet root juice. Water extract.
1	Red.	Rose.	Pink.	Reddish orange.	Orange.	Rose.
2	Rose.	"	"	"	"	"
3	Blue.	Pink.	"	Rose.	Pink.	Red.
4	"	"	Violet.	"	Less pink.	Becoming gradually darker after P_H^+7 until at P_H^+ 10 and 11 it is of a violet or bluish red color.
5	"	Violet (pink-blue).	Blue.	"	Less pink than P_H^+ 4.	
6	Blue-green.	Violet (pink-blue).	Blue-green.	Violet.	Less pink than P_H^+ 5.	
7	Green.	Purple.	Green.	Both P_H^+ 7 and P_H^+ 8 pass through pink-violet to bluish, then turn brown. Very rapid changes.	Less pink than P_H^+ 6.	
8	"	Blue-green.	"		Blue at H ion concentration of 4×10^{-8}.	
9	"	"	"		Blue-green to green.	
10		"	"	Reddish brown.	Green.	
11		"	(Pink.)	Pink.	"	
12		Green-yellow.		Brown.	Yellow.	Orange-brown.
13		Golden "		Green.	"	Yellow.

*Extracted with 95 per cent alcohol—$CHCl_3$; evaporated to dryness; the residue dissolved in water; water extract shaken with CCl_4; evaporated to dryness; redissolved in water for use as indicator; colors stable in buffer solutions after standing 4 days.

The colors were observed under a light of constant and excellent quality from a recently described lamp.[6] It was not considered desirable to attempt a more accurate description of the colors because of the considerable degree of variation dependent upon the strength of the indicator. In radish the indicator was very dilute so that shades of red varied to shades of pink. Some petals, such as those of pansy and violet, contain very large amounts of anthocyan in comparison with others (such as light blue hyacinth, *Browallia speciosa*, etc.) and in extracts of the former the colors observed with buffer solutions more nearly match the normal color of the cells from which the indicator was extracted, as regards the intensity of the color. The number of drops of indicator and the number of cc. of buffer solution used were always such that the results for a single indicator are comparable at the various concentrations of hydrogen ion.

When crude extracts of the pigment are used, the indicator often decolorizes so quickly that it is necessary to filter as rapidly as possible (or the filtration may be omitted entirely if necessary). When extracts were somewhat purified the indicator remained more stable, even for long periods, which may indicate that in crude extracts the decolorization may be due (in part at least) to the action of enzymes. Some indicators when added to buffer solutions of hydrogen ion concentrations in the region of P_H^+ 4 to P_H^+ 6 rapidly decolorize, but upon the addition of very strong acid in sufficient quantity, the color characteristic of the indicator for acid solutions of P_H^+ 2 or 3 reappears. This may indicate that the colorless condition of the indicator may be due to an isomerization of the indicator or the formation of a leuko base.

The results showed very little difference whether crude alcoholic or aqueous extracts of the indicator were used. It seemed very surprising that cranberry juice indicator at P_H^+ 11 should give a pink color, but a redetermination of the buffer solution on the gas chain showed the previous determination to be accurate, and when the indicator was dried on paper it was shown that a drop of buffer solution of a strength of P_H^+ 11 gave a pink color. Red beet juice may be bluish red when slightly alkaline. Hence

[6] Luckiesh, M., *Science*, 1915, xlii, 764.

the pigments of some plant cells (when the indicator is used *in vitro*) may assume a more or less reddish color when alkaline.

It is evident from a consideration of the results that the reaction of the cells studied ranges from about P_H+ 3 to about P_H+ 8.

In a number of cases the color of the flower changes as the flower fades, thus indicating a change in reaction which accompanies the death of the cells. When *Browallia* petals are just expanded they are blue, but when past their maturity they may become somewhat blue-green in certain parts. *Primula obconica* at first has pink petals but later the color changes to a blue and then may become white. In comparison with *Primula obconica* it is well to consider *Primula chinensis*. The petals of *Primula chinensis* are normally red but later become blue, which color is permanent. This blue color results similarly when the petals are removed while red and are placed in a desiccator.

These two *Primula* flowers, by the change of color in the petal cells, show a decrease in acidity when the cells become old or die. The data further indicate that the cells of *Primula chinensis* are still very slightly acid when the cells die. Such small amounts of acid cannot be detected by ordinary titration methods. If we call the buffer solutions acid up to P_H+ 7, neutral when at P_H+ 7, and alkaline when higher than P_H+ 7, it is evident from the experiments that it is unsafe to call cell sap acid when red, neutral or alkaline when blue, and markedly alkaline when green, unless the color changes of the particular pigment are first studied by some method such as that of using buffer solutions of known hydrogen ion concentration.[7]

Mann considered that the coloration of the anthocyan in the seed coat of cow-peas[8] indicated acid when rose or purple and alkali when blue or black, and that both the alkaline- and the acid-reacting anthocyanine may occur in the same cell. The writer was unable to find in microtome and hand sections of cow-peas (of different colors), fresh from immature and mature pods, any cases where the same cell contained both red and blue pigments before drying. The cells were mounted both dry and in water. It may be added that if the color comes out in water it indicates that the cells are dead.

[7] Crozier, W. J., *J. Biol. Chem.*, 1916, xxiv, 443.

[8] Mann. A., *J. Agric. Research*, 1914, ii, 39.

In this connection the writer has made observations on several tropical flowers (*Bromeliaceae*) which have a blue pigmented portion merging into a red pigmented part. In fresh material no cells were found to contain both red and blue colors unless the section became dried, in which case one end of the red cell became blue and the blue color progressed along the cell until all of the cell was blue. Fresh material showed cells containing red pigment to adjoin cells containing blue pigment. The fact that blue cells never became red indicates that the blue pigment represents a decrease in the acidity of the cell sap. At P_H^+ 8 the pigment extracted from both red and blue parts gave a bluish green coloration with buffer solutions, while at P_H^+ 9 the pigment extracts gave a green coloration with the buffer solutions. It is evident, therefore, that the blue color of a pigment is insufficient as a criterion of the reaction of the cell unless the pigment is first calibrated by solutions of known hydrogen ion concentration.

SUMMARY.

1. In view of the widely accepted idea that the reaction of living cells must be neutral or nearly so in order that life may go on in a normal manner, it is of interest to find normal cells in which the reaction of the cell sap is decidedly acid (P_H^+ 3).

2. The prevailing opinion that the blue color of living cells always indicates an alkaline reaction is erroneous. It is found to indicate a decidedly acid (P_H^+ 3) to neutral reaction (P_H^+ 7), or sometimes barely alkaline (P_H^+ 7 to 8).

3. A considerable change of reaction may occur in the cell as it dies (from P_H^+ 3 to P_H^+ 7).

THE INFLUENCE OF BILE ON AUTOLYSIS.

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While it is common knowledge that bile possesses very decided toxic properties, the literature on this subject leaves much to be desired in regard to the nature and mode of action of the active constituents. The substances which have been accredited as responsible for the toxicity are accordingly (1) the bile salts, sodium glycocholate and sodium taurocholate; (2) cholesterol; (3) bile pigments; and (4) lecithin. The possibility cannot be overlooked that the various groups of investigators, though apparently reporting conflicting results, are yet partially correct, depending upon the criteria used for their interpretations. It is quite probable that the different constituents of bile may act upon the various body structures quite differently, and thus be responsible for the diversity of opinion as to which is and which is not the toxic constituent.

It has long been known that bile and bile salts have a powerful cytolytic action (Rywosch (1)) though the mechanism of this action does not appear, to my knowledge, to have been clearly defined. It is with such a problem that this paper is concerned.

Small blocks of various organs were taken as soon as possible after killing an animal, placed in the freshly withdrawn bile, then allowed to stand in the incubator at 38°C. from 2 to 4 hours. The bile was then poured off, and the tissues fixed in Zenker's solution. Sections were prepared in paraffin and stained with hematoxylin and eosin. There is found to have occurred a very marked alteration at the periphery of the tissues, while the central portions are unchanged. In the peripheral margins, the nuclei have lost their capacity for staining with hematoxylin,

even losing all evidence of their former position or form. The cytoplasm takes the eosin stain, but most peripherally becomes pale or even disappears altogether, leaving merely the supporting connective tissues. Within, the nuclei are pyknotic, forming a narrow zone of dense blackened nuclei just without the central, normally appearing, unaffected area.

The depth of the changed cortical area depends upon several factors. These factors appear to be the penetration of the cytolytic agent, the character of the tissue, and the alteration produced in the affected zone. In regard to actual depth of action, it may be stated that nuclei in several specimens of series running for 4 hours had completely disappeared throughout in tissues 0.3 to 0.4 cm. in diameter. Consequently I lost many specimens because they were too thin, or were left too long in the incubator, before I was fully aware of the rapidity of the reaction.

The question then arose, what is the significance of this marked cell change? It is generally stated that bile or its salts have in themselves no digestive action, except perhaps a slight action on carbohydrates, but here is evidence of marked alteration, even of protein material. If the cytolytic action is a solvent process, it should act upon fibrin and other pure proteins, which it does not do. The reasonable explanation appeared to be that the autolytic processes of protoplasm are markedly accelerated by bile or certain of its constituents, and consequently it was found that no alteration could be demonstrated in the tissues, if previously to incubation in the bile they were killed by immersion for a minute in boiling water. This condition obviously is now comparable to the negative results obtained on precipitated proteins. Here the conditions necessary for autolytic processes are destroyed and hence the bile cannot exert its cytolytic action. Further evidence of this view of the action of bile is obtained in the following experiment.

Tissues were immersed in bile, and placed, respectively, in the incubator at 38°C.; on the laboratory table at 25°C.; and in the ice box, at 0°C. After 2½ hours the tissues were dropped into Zenker's fluid and finally sectioned. On examination it was found that practically no action had occurred in the cold specimen, considerable in that at 25°C., and most in that kept at the optimum temperature of 38°C. This follows the law of enzyme action with respect to the influence of temperature. Further evidence of autolysis is the fact that those tissues most rapidly autolyzing

post mortem are those that are most affected by bile salts, namely, in order, adrenal, spleen, liver, thyroid, heart muscle, smooth muscle, voluntary muscle, and lastly, connective tissue, which is not affected.

Each block of tissue presents its own control, since the interior is unaltered, though under the same temperature influence. However, separate controls were studied in Ringer solution, in chloroform-Ringer, and in serum. In none of these instances did there occur the slightest evidence of autolysis during the time of incubation, 2 to 4 hours. In serum and alkaline Ringer solutions no autolysis could be expected, since serum (5) is known to be protective against autolysis and against hemolysis of erythrocytes, and alkalies (6) are also known to inhibit autolysis. According to some investigators chloroform-Ringer (7) should have given positive evidence of autolysis, but these authors allowed a longer duration of autolysis than that used in my bile experiments. Hence, while chloroform may be a real accelerator of autolysis, it is extremely weak compared to bile or bile salts. The same is true for acid solutions (8) which have been found to accelerate autolysis, but they are also extremely weak compared to bile action. As an illustration of this, on comparison of the effect of 5 per cent bile in Ringer solution on a specimen of fresh thyroid gland it was found to be very much greater than the effect of 0.05 N or 0.025 N HCl on another specimen of thyroid gland. It will be recalled that the optimum concentration of acid for autolysis (Bradley (8)) is near 0.05 N acid. I have repeatedly found that diluted bile is considerably less active than whole bile. While I cannot say, from the criterion employed, that 5 per cent bile is one-twentieth as active as whole bile, yet the effect of the optimum concentration of acid is very much below this 5 per cent bile; consequently the differences in activity are in all probability greater than the rough estimate I have given.

End-points are not readily determinable by this technique, consequently only alterations in coagulable proteins that are subject to morphological differentiation as occurring in cell structures are used as criteria of rate and degree of autolysis.

In order to ascertain, if possible, the relative importance of the constituents of bile, some analytic experiments were carried out. Gall bladder bile is known to vary considerably in its quantitative relations, consequently it was deemed advisable

to determine the amount of bile salts in a particular sample of bile and then make up solutions of commercial bile salts to the corresponding concentration.

Sheep bile was obtained fresh from a near-by abattoir. A portion was placed in the ice box till analysis and special treatment of the other portions were completed. On analysis, according to the method given by Hoppe-Seyler (9), this specimen of bile was found to contain 10.44 per cent of bile salts. This figure was used as a basis of subsequent preparations in this experiment.

To a third portion an equal volume of animal charcoal was added and it was then evaporated to near dryness over the water bath. This was extracted repeatedly with alcohol and each extraction filtered. The pale yellow extract was then evaporated to dryness over the water bath, when the residue was taken up by distilled water. By this means, most of the pigment and all of the mucin were removed.

For autolytic tests, all solutions, unaltered bile, decolorized mucin-free bile, and solutions of sodium glycocholate and of sodium taurocholate were made up to 40 per cent of the original concentration as determined. Thus whole bile was made up to 40 per cent, as was the decolorized mucin-free bile. Sodium glycocholate[1] was made up to 40 per cent of the original salt concentration of bile, which made a 4.18 per cent solution. The sodium taurocholate[1] was marked "47.5 per cent," consequently sufficient salt was weighed out to make a 4.18 per cent solution. Thus I was able to compare the action of equally concentrated solutions of bile, of decolorized mucin-free bile, sodium glycocholate, and sodium taurocholate.

Approximately equal sized portions of absolutely fresh rabbit liver were placed in each of the four solutions and incubated at 38°C. for 2 hours. The tissues were then killed by Zenker's fixative.

The least penetration, as measured by the position of the zone of pyknotic nuclei, occurred in the specimen in sodium glycocholate, though the cytoplasm of the marginal cells had undergone considerable solution. Sodium taurocholate penetrated to a greater depth, equal to that of bile solutions, but no solution of cytoplasm appears to have occurred. The decolorized mucin-free bile was practically similar in action to the solution of sodium

[1] Merck and Co.

taurocholate, while the greatest amount of solution of cytoplasm occurred in the specimen immersed in unaltered bile.

In this experiment it is found that whole bile has a total activity greater than that produced by corresponding concentrations of the bile salts[2] taken separately. It is also greater than that of bile treated with animal charcoal and alcohol. The character of the active constituents lost by this process I have not determined. The statement can be made, however, that whole bile contains a mixture of active constituents whose total activity is not equaled by bile treated with animal charcoal and alcohol, nor by corresponding concentrations of separate solutions of either of the bile salts. Further, the two bile salts appear to behave somewhat differently, the sodium glycocholate having the greater solvent effect on cytoplasm. This may be taken to indicate a degree of specificity of particular accelerators for autolytic processes.

No precautions were taken to prevent bacterial action. The reaction is much too rapid to be ascribed to bacterial decomposition since in control experiments without bile no comparable changes are produced. Sellards (5) found necrosis to occur in the salivary glands in the living animal when injected aseptically with bile or bile salts; and furthermore, the results of the experiments of Bunting and Brown (11) on necrosis following intraperitoneal injections of bile are not likely to be explained by bacterial contamination. Boiling the bile previous to its application to tissues does not diminish its activity, while no evidence of activity is found when boiled tissue is treated with unboiled bile.

The conclusions drawn from the work here presented are the following.

1. Bile is a powerful cytolytic agent.

2. This cytolysis is produced by virtue of the co-enzyme or activating action of the constituents of bile on the autolytic enzymes or processes.

[2] According to Long and Johnson (10) the various commercial preparations of bile salts are subject to rather wide variations in actual concentration of the pure bile salts; consequently the comparisons will necessarily be held in some reserve.

3. Whole bile is more powerful though less penetrating than corresponding concentrations of either of the bile salts.

4. Bile boiled down with animal charcoal and treated with alcohol has lost a portion of its activity.

5. The maximum effects of bile or bile salts are many times greater than the effects of the optimum concentration of acids.

6. Sodium glycocholate appears to affect cytoplasmic material more than does sodium taurocholate, though both affect nuclear material about equally.

7. These facts, correspondingly, have to be considered in any study of the toxicity of bile, and may center attention on alterations in cellular metabolism produced in such intoxications, and furthermore, this agent may be of service in the study of the fundamental factors of autolysis.

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THE RATE OF UREA EXCRETION.

II. THE RATE OF EXCRETION OF ADMINISTERED UREA IN YOUNG HEALTHY ADULTS ON A CONSTANT DIET.

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This study of the rate of excretion of urea administered to young healthy adults who were on a constant diet was carried through in order to obtain detailed information as to the range of variation in the function of the normal kidney under uniform conditions. The work of Schlayer and others on water and chloride excretion by the kidneys has been interpreted as indicative of a wide variability in kidney action even under the same conditions. This variation is assumed to arise from the inconstancy of what is termed the *Reizzustand* of the organ. On the other hand, Ambard believes that the kidney manifests no intrinsic variability in function. He maintains that if the concentration in the blood and in the urine of the substance excreted is known, the action of the normal kidney may always be predicted with mathematical accuracy.

We have found in the literature only two single experiments in which the rate of urea excretion after urea administration has been determined in man by dependable methods. Wolf[1] and Cathcart and Green[2] state that urea taken by mouth is quantitatively excreted within 24 to 48 hours, and Wolf showed that the rate of excretion was greatest in the first few hours after the ingestion of the urea.

Achard and Paisseau,[3] McCaskey,[4] and Pirondini[5] showed that results which were of value in the detection of functional abnormalities in renal

[1] Wolf, C. G. L., *Biochem. Z.*, 1912, xl, 234.

[2] Cathcart, E. P., and Green, H. H., *Biochem. J.*, 1913, vii, 1.

[3] Achard, C., and Paisseau, G., *Compt. rend. Soc. biol.*, 1904, lvi, 894.

[4] McCaskey, G. W., *Med. Rec.*, 1914, lxxxv, 507.

[5] Pirondini, E., *Policlinico Sez. Chir.*, 1915, xxii, 259, 326, 398.

disease may be obtained even when the unreliable hypobromite method of urea estimation is used.

The general plan we adopted was to measure the rate of excretion during a fore-period of 3 days, and during a period of 3 successive days on which 20 or 40 gm. of urea were administered. The urine was collected every 4 hours during the day, and a 12 hour collection was made at night.

The subjects of these experiments were instructors, internes, and students. The ages were between 20 and 35 years. All were in apparent health.

Methods of Urea Estimation.

We had already planned and commenced this work, using Folin and Farmer's microchemical methods of nitrogen determination in the blood and urine, before Marshall's description[6] of the urease method appeared. But after we had confirmed the accuracy of his method we used it exclusively, both for urine and blood analyses. It is unnecessary to give any of our figures in substantiation of the soundness of the principle of the method, since this has already been amply established. But we had to discard our earliest results until we had adopted the two following modifications in working with urine:

1. At first we used 10 per cent thymol in chloroform as a preservative for the urine. We noted occasionally that the amount of urea found was less than the expected quantity, in spite of the fact that duplicate estimations checked perfectly. These were always cases in which the urine was alkaline in reaction. Part of the urea had already been converted into ammonium carbonate. In any neutral or alkaline urine this change takes place with great rapidity, at least in any laboratory where urine is a material which is commonly worked with. It is due to the action of urease-containing organisms. The usual urinary preservatives, such as thymol, chloroform, and toluene, all of which we tried, are not always able to prevent their action. Acting on Armstrong and Horton's[7] finding that the action of urease is absolutely inhibited by any concentration of H ions much above that found at the neutral point for litmus, we thereafter had the urine passed directly into vessels containing 20 cc. of N H_2SO_4 for each 4 hour collection. Except in the case of patients with an infection of the urinary passages, in which some urease-producing organism is present which decomposes the urine before it leaves the bladder, we have never since then had any trouble from this cause.

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[6] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xiv, 283.

[7] Armstrong, H. E., and Horton, E., *Proc. Roy. Soc.*, 1912, lxxxv, 109.

2. Another very necessary modification is the neutralization of the urine before adding the urease extract. Some urines are sufficiently acid to destroy such a considerable part of the ferment before it can commence to form ammonium carbonate that the urea present is not completely converted. We discovered this early in our work, since we had adopted the plan of running duplicate estimations to one of which a known amount of urea was added. An incomplete recovery of this urea, when a corresponding quantity of urea in water was completely accounted for, could not be due to anything but some inhibitory substance in the urine. This inhibition only occurred with acid urines and was completely removed by bringing the urine to the neutral point for rosolic acid. This indicator was chosen, since it changes color just about the point of absolute neutrality.

All the estimations reported here were carried out after the adoption of these two precautions—the collection of the urine in N H_2SO_4, and its subsequent neutralization to the neutral point for rosolic acid.

The details of the technique used for urine were as follows: The specimens of urine were measured in graduated cylinders. They were then poured into volumetric flasks and diluted to the mark with water, so that about 10 cc. of 0.1 N HCl would be required to neutralize the ammonium carbonate formed from 5 cc. of urine. This is accomplished by diluting the 4 hour specimens of the first 3 days to 500 cc., and the night urine to 1,000 cc. On the 4th, 5th, and 6th days when urea is taken, the 4 hour specimens were diluted to 1,000 cc. and the night specimen to 2,000 cc. This high and nearly uniform dilution makes the end-points much easier to recognize.

5 cc. of urine were pipetted from each specimen into each of three flasks, and about 100 cc. of distilled water and two drops of rosolic acid were added to each. All were brought to a first faint red color by adding the same amount of 0.1 N NaOH. 2 cc. of an aqueous extract of freshly powdered soy bean was pipetted into two of the flasks, and after the addition of a few drops of toluene and a known quantity of urea to one, they were corked and left until the following day. To the remaining flask, methyl orange was added and it was titrated at once with 0.1 N HCl until the first tinge of pink developed after the disappearance of the red color due to the rosolic acid. The amount of 0.1 N HCl required was noted, and to it was added the acid required to bring 2 cc. of the urease extract in 100 cc. of water to the same color. Next morning, using the same indicator, the two fermented flasks were titrated to the neutral point with 0.1 N HCl. The acid was run in quickly until the red of the rosolic acid had disappeared. Thereafter it was added slowly, watching for the appearance of a cloudiness which results from the precipitation of some substance in the soy bean extract. This occurs just before the end-point is reached and tends to obscure it somewhat. We found that we were helped in deciding as to the exact end-point if we observed the color of an added drop of methyl orange before it diffused through the fluid. A deep reddish brown color is given if sufficient acid has already been added. The flasks should not

be employed for any other purpose. Even slight traces of silver or copper salts interfere markedly with the action of the ferment. Solutions of urea of known concentration prepared from urea purified by repeated crystallization were estimated each day to check the activity of the soy bean extract.

The Diet.

The diet was constant as regards nitrogen, but provision was made for some degree of elasticity in the amount of carbohydrate taken. Each meal was well within the capacity of a moderate appetite. After it was finished a nitrogen-free cornstarch paste was served. When taken with a spoonful of jam and the small amount of cream which was provided, this made a pleasant dessert. If the subject were not hungry he could leave it out, taking only the cream, and if he happened to be hungry he could take it all. As a matter of fact all the subjects took some of this cornstarch, and nearly all of them took it all, so that the variation in water intake from this cause was not great.

The meals at 8 a.m., 4 p.m., and 8 p.m., were similar and consisted of one egg, 50 gm. of bread, 10 gm. of butter, and 5 gm. of cocoa mixed with 240 cc. of milk. The 12, noon, meal contained 150 cc. of Campbell's tomato soup, neutralized with $NaHCO_3$, and heated with an equal quantity of milk, 50 gm. of bread, 10 gm. of butter, 50 gm. of potatoes, and one egg. 180 cc. of water were taken with this meal, and this was the only water allowed, as such. A little NaCl was given with the egg.

The total water content of the diet (including the 180 cc. of water and the water in the cornstarch) was about 1,710 cc. The protein content was 75.9 gm., the fat 120 gm., and the carbohydrate 220 gm., including the cornstarch.

We are much indebted to Miss Sloan, the dietitian of the Stanford Medical School, for the care she took in weighing and preparing this food.

The Total Urea Excretion.

In Table I, the averages and limits of variation in the total urea excretion for each day of the diet are given. These are compiled from the results obtained in thirty individuals who took 20 gm. of urea on the 4th, 5th, and 6th days of the diet.

The effect of the variable quantities of nitrogenous food taken before the diet was begun does not last beyond the 1st day of the diet. In Table I, the averages for the 2nd and 3rd days are practically identical. In cases to whom no urea was given, the average for the 2nd day did not differ appreciably from those of the subsequent days. Thus in six subjects on the diet, who took no urea, the average 24 hour urea excretion for 6 successive

TABLE I.

Averages and Limits of Variation of the Total 24 Hour Urea.

Fore-period.

1st day.			2nd day.			3rd day.		
Average.	Highest.	Lowest.	Average.	Highest.	Lowest.	Average.	Highest.	Lowest.
17.40	22.8	12.9	15.78	19.2	10.7	15.45	19.5	9.4

Urea period.

20 gm. of urea at 8 a.m. each day.

4th day.			5th day.			6th day.		
Average.	Highest.	Lowest.	Average.	Highest.	Lowest.	Average.	Highest.	Lowest.
32.27	35.5	28.1	34.37	40.7	30.0	34.53	40.5	29.5

days on the diet amounted to 16.8, 15.7, 15.9, 15.9, 14.9, and 15.1 gm. With the diet we used, therefore, an equilibrium is reached on an average within 24 hours.

The averages during the urea period show that the 20 gm. of urea administered are not quite completely excreted. There is a retention of a few gm. of urea which can be accounted for if the diet is continued for another day without giving urea. It is also noteworthy that there is a slight rise in the average excretion on each day during the urea period.

The range of variation amounts to about 10 gm. a day and is quite as large in the fore-period as in the urea period. The main cause of this wide variation becomes apparent when the data are arranged in order of magnitude. It is then seen that the higher values are derived throughout from one group of individuals, and the lower from another. In Table II all the urea excreted while the diet was taken with the exception of the urea of the first day is given for each individual.

The range of variation in Table II is due to some subjects having on every day of the diet a higher urea excretion than others. This explains the range of variation in Table I, for there the highest urea excretions for each day are derived almost without exception from the subjects BO$_1$ and Ad, who excreted more

TABLE II.

The Total Urea Excreted Excluding the 1st Day.

(Arranged in order of descending magnitude.*)

No.	Subject.	Urea.	No.	Subject.	Urea.	No.	Subject.	Urea.
		gm.			gm.			gm.
1	Bo_1	148.5	10	G	137.8	19	Ai_2	127.2
2	Ad	142.9	11	S_1	137.2	20	Boy_1	126.4
3	C	140.8	12	F	136.1	21	Br_2	126.3
4	Br_1	140.2	13	Cr	134.9	22	B	125.6
5	Wa	138.9	14	J	133.4	23	Add	124.5
6	P_2	138.1	15	S_2	131.2	24	K_2	124.2
7	K_1	138.0	16	P_1	130.7	25	We	123.8
8	D	138.0	17	Je	127.8	26	Ai_1	121.2
9	Boy_2	137.9	18	K_3	127.6	27	M	110.4

* The totals of Bo_2, O, and W cannot be given, since one collection of urine was lost in each of these cases.

than 140 gm. of urea in 5 days, and the lowest urea excretions for each day come from the subject M, who excreted only 110 gm.

These constant differences in the level of urea excretion are certainly not to be ascribed to corresponding differences in the capacity of the kidneys of these subjects in excreting urea. If that were the case, the subject M would have had a high concentration of urea in his blood and tissues on account of very marked urea retention. But as a matter of fact, the blood urea concentration of M on the 3rd and 6th days of the test was only 0.0156 and 0.0558 per cent, while the blood of BO_1 on these days had a concentration of 0.0261 and 0.0672 per cent. The subject M, who excreted the lowest amount of urea, had on the 3rd day of the diet the lowest blood concentration we have observed.

The cause of these different levels in urea excretion lies in the existence of different levels in the rate of protein catabolism. Although M took as much protein as BO_1, he did not excrete so much urea, because in his case presumably the amino-acids were in great part utilized in protein synthesis. In the case of BO_1 on the other hand, a much larger proportion was deaminized, with the resulting formation of larger amounts of urea.

The variable factor introduced by the existence of these different levels of protein catabolism in different individuals must be eliminated if we wish to compare the work of their kidneys

in excreting preformed urea. This will be accomplished if we accept the average excretion of the 2nd and 3rd days of the fore-period as a measure of the level of protein catabolism for each subject, and subtract this value from his urea excretion on the days when urea was given. The difference gives the rate of excretion of the preformed urea which was given at the same time and in the same manner to all. For convenience we have termed the results of this subtraction "administered urea excretion."

The Degree of Variation in the Rate of Excretion of Administered Urea.

In Table III the rate of excretion of administered urea thus calculated is given for each subject for each day of the urea period along with the average excretion of each subject for that whole period. The average excretion of the fore-period is also given. Each column is arranged in order of descending magnitude, so that some idea of the degree of dispersion may be obtained at a glance.

Before the significance of these figures in Table III as indicative of functional variation in the work of the kidney can be appreciated, it is necessary to obtain some conception of the variation which might be introduced through the error in our method of calculating the amounts which we have termed administered urea excretion. For, although by taking the average excretion of the fore-period as representing the level of protein catabolism and subtracting it from the total excretion after urea administration, we have eliminated an inconstant factor—the variable rate of protein catabolism in different individuals—we cannot thus obtain a mathematically accurate representation of the rate of work of the kidney in excreting the administered urea. For there are not only differences in the rate of protein catabolism in different individuals; there are also smaller though not inconsiderable fluctuations from day to day in the same individual. These individual variations are not taken account of in our figures, since they are obtained by a method which assumes that the amount of urea obtained on the 2nd and 3rd days of the diet remains constant in each individual throughout

the 4th, 5th, and 6th days, whereas it is a universal experience that even under the most constant conditions urea excretion does not remain absolutely uniform in any individual. Folin[8] for instance found a variation of 5.6 to 8.1 gm. of urea within a few days in the same individual on his starch-cream diet.

A simple inspection of the arrangement of the subjects in Table III in the fore-period as compared with the urea period strongly suggests that we have failed to measure accurately the rate of protein catabolism. Those subjects who have the highest excretion in the fore-period have very often the lowest excretion of administered urea, and *vice versa*. It would seem that we have overestimated the rate of protein catabolism in some and underestimated in others, an error which introduces a false variability in the calculated rates of excretion of administered urea.

It is probable therefore that in reality the rate of excretion of administered urea by the normal kidney has a greater uniformity than that recorded by our figures, since they are influenced not only by the possible variability of kidney action, but also by the known variability in each individual from day to day of all those extra-renal factors which are conveniently grouped under the term "protein catabolism."

In order to obtain a conception of the true variability of kidney function in dealing with administered urea, we must compare a group of subjects who took no urea with a group of those who did. If it were found that there was a greater degree of variability in the urea group, it would be permissible to ascribe the increase in variability to inconstancy in the rate of excretion of administered urea by the kidneys.[9] The difference in the variability of the two groups would be a measure of the variability of kidney function.

We accordingly placed six subjects under the same conditions observed for the urea group recorded in Table III, except that they took no urea. Any differences between the individual averages of the 2nd and 3rd days and the amounts of urea excreted on the 4th, 5th, and 6th days, represent the extent by

[8] Folin, O., *U. S. Dept. Agric. Report*, 1911, xciv, 233.

[9] It has been well established, contrary to what might have been expected, that urea has no influence on protein metabolism (see Abderhalden, E., *Z. physiol. Chem.*, 1915, xcvi, 1).

which we would have overestimated or underestimated the amounts of "administered" urea excreted, if they had taken any. These differences for each subject and for each period are detailed in Table IV in the same way as for the urea group. When the average of the 2nd and 3rd days is exceeded, the difference is given as plus; when it is less, the difference is minus.

In comparing the degree of variability of the groups in Tables III and IV, the average deviation is a much better measure to employ than the simple range of variation, since it takes all the variations into account, and not only the most extreme. The average deviation[10] is the average of all the differences between the individual amounts and the average amount for each group. These differences are added without paying attention to plus or minus signs, and the sum divided by the number of individuals in the group gives the average deviation.

These values are given at the foot of Tables III and IV. It will be noted that there is no marked or constant difference in the variability of the two groups at any period. The most representative period of all is the average of the individual 24 hour excretion of the 4th, 5th, and 6th days. The average deviation for this period in the urea group is 1.40, while it is 1.41 for those who did not take urea.

Since there is no appreciable difference in the degree of variability of urea excretion between those who took urea and those who did not, we may conclude that the variability found in the calculated rate of excretion of administered urea may be accounted for by the variation in the rate of protein catabolism from day to day in the same individual. And although we cannot positively assert that there is no variability in the rate of excretion of administered urea[11] yet any variability which may exist must be of slight extent—so slight that we are not able to demonstrate its existence.

[10] Yule, G. U., An Introduction to the Theory of Statistics, London, 1912, 144.

[11] Dr. E. S. Kilgore, who suggested the use of the average deviation in comparing the variability of the groups with and without urea, also pointed out that the "probable error" of these average deviations was such that some variability in the rate of excretion of administered urea could not be definitely excluded.

This result appears to us to be of particular interest on account of the widely prevalent conception of the essential variability of normal kidney function under supposedly constant conditions, a view which is derived from the work of Schlayer and others.

The Percentage of Administered Urea Excreted During Successive Periods of the 24 Hours.

After the ingestion of 20 gm. of urea there is an almost instantaneous increase in the urea concentration of the blood which continues until a maximum is attained about 2½ hours later. Thereafter there is some decrease, and there follows throughout the remainder of the 24 hours a succession of fluctuations at concentrations which all remain above the level observed before the urea was taken. The total urea excretion is also immediately increased, but there is no definite rise and fall from a maximum, and in general no close relationship is evident between the urea concentration in the blood and the rate of urea excretion. The rate of excretion is greatest during the first 8 hours, and thereafter decreases and reaches its lowest level during the sleep of the night. This decrease, though in general constant, may show considerable fluctuations during short intervals. These points are illustrated in the following protocol which records observations made on a subject who took 20 gm. of urea at 8 a.m. while on the diet.

The rapidity of urea absorption is shown by the considerable increase in blood urea concentration as early as 10 minutes after the urea entered the stomach. Thereafter the urea was entering the blood more rapidly than it was leaving it through the kidneys and into the tissues, so that for 2½ hours at least the blood concentration was rising. As the rate of tissue absorption and kidney excretion became greater than the rate of entry of urea into the blood from the alimentary tract, the blood urea concentration gradually fell. The subsequent fluctuation in blood urea concentration is not atypical, for we have made similar observations in other cases. It is interesting to note that the sharp rise in blood urea concentration is not duplicated in the curves of urea excretion or of urine concentration. The greatest rate

TABLE III.

Total urea excreted (fore-period). Average of 2nd and 3rd days.						Administered urea excreted after the ingestion of 20 gm. of urea calculated by subtracting the total urea of the fore-period from the total urea of the urea period.																					
						4th day.						5th day.						6th day.						Average of 4th, 5th, and 6th days.					
24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.		24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.		24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.		24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.		24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.	
Subject.	Urea	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.
	gm.		gm.		gm.		gm.		gm.		gm.		gm.		gm.		gm.		gm.		gm.		gm.		gm.		gm.		gm.
D	18.24	F	10.68	Bo_1	6.88	M	19.94	M	15.75	Ad	11.37	Bo_1	22.87	Bo_1	17.53	Ai_2	13.62	Br_1	24.54	Ai_2	17.66	Ai_2	14.19	Boy_1	20.41	Ai_2	15.90	M	12.92
F	17.97	G	10.11	Ad	6.83	K_2	19.83	K_2	14.43	M	11.26	Ai_2	21.56	B	16.78	Bo_1	13.40	S_1	22.84	S_1	17.51	K_1	13.76	M	20.27	B	15.66	Br_1	11.25
Bo_1	17.87	Ad	9.90	F	6.71	Je	19.26	We	14.24	We	10.94	Boy_1	21.39	M	15.90	W	12.08	Boy_1	22.57	B	16.79	S_1	13.05	Ai_2	20.15	M	15.56	D	11.23
J	17.61	Bo_1	9.89	G	6.57	Ad	18.69	Ai_2	14.21	K_2	10.75	M	20.91	Ai_2	15.83	We	12.08	K_2	21.39	P_1	16.37	D	12.98	Br_1	20.12	Je	14.91	K_1	11.21
G	17.54	J	9.84	J	6.57	We	18.26	Bo_2	13.80	F	10.51	Ad	20.55	Ad	15.70	Br_1	11.83	P_1	21.21	P_2	16.28	Br_1	12.58	K_2	19.87	S_1	14.71	Bo_1	11.20
Bo_2	17.40	C	9.74	Cr	6.51	Wa	18.17	Ad	13.56	Je	10.49	Je	20.54	We	15.56	M	11.59	Ai_2	21.11	Br_1	15.73	O	12.41	Je	19.84	We	14.52	We	11.20
Wa	17.29	Br_1	9.68	W	6.28	Ai_2	17.78	Je	13.53	Br_2	10.19	P_2	19.98	Je	15.53	D	11.43	P_2	20.40	Je	15.68	Br_2	12.29	Bo_1	19.72	P_1	14.32	Br_2	11.10
Boy_2	17.25	D	9.54	P_1	6.19	Cr	17.47	B	13.42	Cr	9.69	K_1	19.74	W	15.41	K_2	11.35	K_1	20.28	Boy_1	15.55	W	11.88	S_1	19.52	Bo_2	14.20	Je	10.98
S_2	17.16	Bo_2	9.24	D	6.15	K_1	17.28	Br_2	12.95	K_1	9.32	Br_1	19.65	K_2	15.23	Je	11.33	M	19.95	O	15.51	M	11.84	Ad	19.50	D	14.12	K_2	10.62
C	17.12	W	9.19	Br_2	6.07	B	17.27	Cr	12.82	Add	9.28	Ai_1	19.41	Ai_1	14.83	Br_2	10.91	Br_2	19.94	D	15.35	Boy_1	11.37	P_2	19.16	K_2	14.03	S_1	10.53
Ad	16.89	Br_2	9.13	Br_1	6.05	P_2	17.10	Add	12.73	D	9.27	We	19.40	Br_1	14.74	K_2	10.89	K_2	19.79	Br_2	15.17	P_1	11.33	K_1	19.10	Bo_1	13.95	Ad	10.43
O	16.65	P_2	9.12	S_2	5.83	Bo_1	16.97	S_1	12.59	P_1	9.17	K_2	19.26	D	14.46	Boy	10.85	J	19.73	M	15.02	Bo_1	11.18	C	18.71	Br_1	13.93	Boy_1	10.38
Cr	16.55	O	9.09	We	5.80	Add	16.81	D	12.54	B	9.09	S_1	19.00	C	14.45	O	10.73	C	19.70	Bo_1	14.95	Je	11.12	P_1	18.24	Ad	13.86	P_1	10.13
P_2	16.14	S_2	9.00	B	5.79	Bo_2	16.80	K_1	12.54	Bo_1	9.02	C	18.72	Boy_1	14.19	Ad	10.57	Bo_1	19.33	W	14.67	We	10.58	We	17.92	Boy_1	13.84	F	10.02
K_1	16.14	Boy_2	8.94	S_1	5.79	C	16.72	F	12.48	Boy_1	8.93	P_1	18.69	Boy_2	14.16	K_1	10.56	Ad	19.26	Bo_2	14.64	K_2	10.44	Br_2	17.86	Boy_2	13.82	B	9.95
Br_1	15.99	We	8.44	O	5.77	S_1	16.72	P	12.45	S_1	8.37	K_2	18.39	P_1	14.13	B	10.44	O	18.33	C	14.46	K_2	10.41	K_3	17.54	Br_2	13.81	Cr	9.77
W	15.93	K_1	8.40	K_3	5.52	F	16.29	Bo_1	12.37	S_2	8.03	W	18.33	S_1	14.03	S_1	10.23	G	18.22	K_2	14.07	B	10.31	Wa	17.51	K_1	13.36	Add	9.28
S_1	15.74	Ai_1	8.39	Add	5.42	Br_1	16.17	Boy_1	11.79	G	8.01	G	18.04	K_1	13.92	P_1	9.89	Wa	17.89	K_3	13.78	S_2	10.19	B	17.50	C	13.33	S_2	9.23
P_1	15.21	P_1	8.13	K_1	5.38	Br_2	15.92	Br_1	11.32	J	7.97	B	17.96	G	13.71	Cr	9.75	Add	17.80	We	13.76	F	9.94	Cr	17.39	Cr	13.00	J	9.08
K_3	15.01	S_1	8.11	Je	5.35	D	15.72	C	11.09	K_3	5.07	Cr	17.89	Add	13.63	Add	9.64	W	17.53	G	13.65	G	9.93	Add	17.37	Add	12.78	G	8.97
Ai_1	14.73	Ai_2	7.69	Boy_1	5.11	Boy_2	15.15	S_2	10.92	Ai_1	4.75	Br_2	17.72	K_2	13.59	J	9.63	B	17.27	Boy_2	13.62	Cr	9.88	Boy_2	17.23	K_2	12.68	K_2	8.95
Br_2	14.56	K_3	7.64	K_2	4.89	S_2	14.88	J	10.82			Bo_2	17.64	Br_2	13.31	F	9.61	Boy_2	16.95	K_1	13.62	J	9.63	G	16.72	G	12.67	Ai_1	8.21
B	14.23	Add	7.55	Ai_2	4.80	P_1	14.81	G	10.65			Add	17.49	Cr	13.24	S_2	9.47	Cr	16.82	Cr	12.95	Ad	9.34	D	16.46	P_2	12.63		
We	14.02	Je	7.51	M	4.40	G	13.90	Ai_1	10.45			O	17.37	J	13.02	G	8.67	D	16.61	F	12.81	Add	8.92	Ai_1	15.85	F	12.29		
Add	13.97	B	7.46			J	13.85	P_2	9.48			D	17.04	O	12.97			We	16.10	S_2	12.78	Ai_1	8.65	F	15.44	J	12.19		
Je	13.66	K_2	7.41			K_2	13.57	K_3	9.04			J	16.53	S_2	12.84			F	15.84	J	12.72			S_2	15.14	S_2	12.18		
Ai_2	13.36	Boy_1	7.23			A_1	13.35					Wa	16.48	P_2	12.12			S_2	15.18	Ad	12.33			J	15.14	Ai_1	11.84		
Boy_1	13.05	M	6.39									S_2	15.36	F	11.58			J	15.03	Add	11.98								
K_2	12.93											F	14.19					Ai_1	14.79	Ai_1	10.25								
M	10.12																												
Average..	15.67		8.74		5.87		16.64		12.40		9.13		18.72		14.39		10.87		18.85		14.47		11.13		18.14		13.71		10.30
Average deviation.....							1.39		1.20		1.17		1.44		1.06		0.90		2.06		1.37		1.22		1.40		0.88		0.86

TABLE IV.

Average urea excreted (fore-period).						No urea ingested. Differences found on subtracting the average urea excretion of the fore-period from the urea excretion of the following period.																					
Average of 2nd and 3rd days						4th day.						5th day.						6th day.						Average of 4th, 5th, and 6th days.					
24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.		24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.		24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.		24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.		24 hrs. 8 a.m.–8 a.m.		12 hrs. 8 a.m.–8 p.m.		8 hrs. 8 a.m.–4 p.m.	
Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.
	gm.		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*
Ch	18.30	Ch	10.14	Ch	6.39	T	+3.37	Ad	+2.58	Ad	+3.03	T	+2.03	Ad	+2.76	Ad	+3.03	T	+2.90	T	+1.28	Ad	+1.05	T	+2.77	Ad	+2.20	Ad	+2.37
St	16.53	St	9.18	St	6.09	Ad	+1.50	T	+0.17	Sh	+0.77	Ad	+1.80	T	+1.43	T	+1.41	Ad	+1.08	Ad	+1.26	T	+0.84	Ad	+1.46	T	+0.96	T	+0.85
Ad	16.08	Ad	8.82	We	5.85	We	−0.40	Sh	+0.15	Ch	+0.36	We	−1.57	St	−0.12	St	+0.15	Ch	+0.12	Ch	−0.30	Ch	+0.48	We	−0.73	Sh	−0.59	We	+0.13
Sh	15.87	We	8.80	Ad	5.55	St	−1.23	Ch	+0.03	T	+0.30	Sh	−1.98	We	−0.61	We	−0.09	We	−0.23	Sh	−0.69	Sh	−0.37	Sh	−1.92	We	−0.74	Ch	+0.08
We	14.68	Sh	8.28	Sh	5.23	Sh	−1.92	We	−0.46	We	+0.21	St	−2.17	Sh	−1.23	Sh	−0.49	Sh	−1.86	We	−1.15	St	−0.39	Ch	−2.17	St	−0.74	Sh	+0.03
T	13.12	T	7.12	T	4.68	Ch	−2.93	St	−0.90	St	−0.27	Ch	−3.69	C.	−2.19	Ch	−0.60	St	−4.29	St	−1.20	We	−0.51	St	−2.43	Ch	−0.82	St	−0.17
Average..	15.76		8.72		5.63		−0.27		+0.26		−0.73		−0.76		+0.01		+0.56		−0.38		+0.13		+0.18		−0.50		+0.04		+0.55
Average deviation.....							1.62		0.60		0.69		1.44		1.38		0.72		1.50		0.85		0.43		1.41		0.97		0.65

TABLE V.

Time at which blood was collected.	Blood urea concentration per 100 cc.	Hourly urea excretion.	Hourly volume of urine.	Urine urea concentration per 100 cc.	Times between which urine was collected.	Remarks.
	gm.	*gm.*	*cc.*	*gm.*		
7.30 a. m.	0.0300	0.91	143	0.64	7–8 a. m.	Rose from bed at 7 a. m.
8.10 " "	0.0427					At 8 a.m. began drinking 360 cc. water with 20 gm. urea. At 8.10 breakfast.
		1.78	193	0.92	8–9 " "	
8.20 " "	0.0473					
8.40 " "	0.0517					
9.07 " "	0.0547	2.14	109	1.97	½ 9–10 " "	
9.30 " "	0.0577					
10.30 " "	0.0682	2.17	78	2.78	10–11 " "	
11.30 " "	0.0607	1.89	69	2.75	11 a.m.–12 n.	
12.30 p. m.	0.0514	2.00	79	2.53	12 n–1 p.m.	At 12 n. lunch.
1.30 " "	0.0495	2.06	104	1.99	1–2 p.m.	
	[illegible]	2.92	245	1.20	2–3 " "	
3.30 " "	0.0585	1.71	107	1.59	3–4 " "	
		1.45	67	2.17	4–5 " "	At 4 p.m. afternoon meal.
5.30 " "	0.0495	1.70	71	2.38	5–6 " "	
		1.83	85	2.15	6–7 " "	
		1.68	91	1.84	7–8 " "	
8.30 " "	0.0346	1.20	51	2.35	8–9 " "	At 8 p.m. evening meal.
		1.80	75	2.40	9–10 " "	
		0.76	48	1.58	10 p.m.–7 a.m.	Sleeping from 10.30 p.m.–7 a.m.
8.00 a. m.	0.0540	1.58	107	1.48	7–8 a.m.	Rose from bed at 7 a.m.
		0.99	51	1.95	8–9 " "	

of urea excretion corresponds with the maximum volume of urine, not with the highest blood concentration.

The problems raised in connection with the relationship between the rate of urea excretion and the blood urea concentration will be dealt with in a subsequent communication. We refer to the blood here only to emphasize the fact which has already

been demonstrated by Marshall and Davis,[12] that administered urea is not heaped up in the blood, but exists for the greater part in the tissues and is gradually withdrawn from them for excretion by the kidneys. But the relatively high degree of uniformity in the rate of passage of the administered urea through the kidneys is not apparent in the figures of the total urea excretion. These figures show irregular fluctuations from hour to hour, due in the main to the varying rate of formation of urea from food and tissue protein. By subtracting the average total excretion of the 2nd and 3rd days from the excretion of the urea period, as has been done in determining the degree of variability in the rate of excretion of administered urea, we eliminate the differences which exist between different subjects, although individual variations will still prevent more than an approximation being reached. Nevertheless it would seem to be advisable to express the calculated rate of excretion of administered urea during different periods of the 24 hours in terms of a percentage of the 24 hour excretion of administered urea, because such an expression may prove of value as a standard of comparison in detecting functional abnormalities of the kidney.

It has long been known that the amounts of the various constituents of the urine tend under normal conditions to be excreted in larger proportion during the day than during the night; it has also been frequently observed that this relationship may be reversed when the kidneys are diseased, so that the night quantities are greater than those of the day—a condition known clinically as nocturia. The current theory as to the reason for nocturia implies that it arises from an inability on the part of the kidneys to overtake all the extra work thrown on them during the day, so that part of the excretory products are retained to be eliminated during the night. If this explanation be correct, our procedure should provide conditions for the revelation of any latent tendency in this direction, for it involves a marked and sudden call for increased work by the kidneys at the commencement of the day period.

We have therefore worked out for each subject the percentage of the 24 hour excretion of administered urea eliminated during

[12] Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.

the day and night periods, and also during the first 8 hours of the day, the period throughout which the maximum rate of excretion is found. In Table VI these percentages are given for the individual average excretions of the 4th, 5th, and 6th days.

TABLE VI.

The Percentage of the 24 Hour Excretion of Administered Urea Eliminated During Successive Periods of the 24 Hours.

(Calculated from the averages of the 4th, 5th, and 6th days).

8 hrs. of day. 8 a.m.–4 p.m.		12 hrs. of day. 8 a.m.–8 p.m.		12 hrs. of night. 8 p.m.–8 a.m.	
Subject.	Percentage.	Subject.	Percentage.	Subject.	Percentage.
D	68.4	D	85.7	P_2	34.0
F	65.0	We	81.0	Boy_1	30.2
M	63.7	J	80.4	Br_1	30.6
We	62.5	S_2	80.3	K_1	30.1
Br_2	62.2	Boy_2	80.2	Bo_1	29.3
S_2	60.9	F	79.5	K_2	29.3
J	59.9	B	79.4	Ad	29.0
K_1	58.8	Ai_2	79.0	C	28.7
Bo_1	56.8	P_1	78.5	Add	26.5
Cr	56.4	Br_2	77.4	Ai_1	25.2
Br_1	56.0	M	76.7	Cr	25.2
P_1	55.6	G	75.7	Je	24.8
Je	55.4	S_1	75.4	S_1	24.6
S_1	54.1	Je	75.2	G	24.3
K_2	53.5	Cr	74.8	M	23.3
G	53.5	Ai_1	74.8	Br_2	22.6
Ad	53.5	Add	73.5	P_1	21.5
Add	53.4	C	71.3	Ai_2	21.0
Ai_1	51.8	Ad	71.0	B	20.6
K_3	51.0	K_2	70.7	F	20.5
Boy_1	50.8	Bo_1	70.7	Boy_2	19.8
		K_1	69.9	S_2	19.7
		Br_1	69.4	J	19.6
		Boy_1	67.8	We	19.0
		P_2	66.0	D	14.3
Average..	57.1		75.5		24.5

In every case more than one-half of the 24 hour excretion of administered urea appears during the first 8 hours, and there is

not more than about one-third at most excreted during the 12 hours of night. The average day excretion is more than three times greater than that of the night.

The existence of a condition described as "kidney fatigue" has been hypothecated by some observers[13] to explain certain experimental results. Whether this view is justified or not, we certainly have no evidence of any such condition in these experiments, although the addition of a heavy load on the work of the kidneys for 3 successive days might be expected to elicit such a state. On the contrary we find that the average percentage excreted during the first 8 hours after urea administration increases slightly, from 54.9 per cent on the 4th day, to 58.3 per cent on the 5th day, to 59.3 per cent on the 6th day.

Urea taken by mouth by virtue of its extreme diffusibility is quickly and evenly distributed throughout all the tissues of the body. As the urea content of the blood is lowered by the secretory activity of the kidneys, the urea is gradually returned from the tissues to the blood, and is excreted. The constancy in the rate of excretion of administered urea shows that there must be a relationship at any one period of the 24 hours between this rate of excretion and the amount of the administered urea still remaining in the body. The exact range of variation in this relationship will of course not be exactly determinable because of the individual variations in protein catabolism, but the averages for the different periods should give a close approximation. This relationship, expressed in the form of the percentages of the administered urea within the body which were excreted at successive 4 hour intervals during the day, is given in Table VII.

There seems to be a fairly constant relation between the rate of excretion and the amount of administered urea in the body. The lowest percentages are found during the first 4 hours of each day. This might have been ascribed to the fact that the 20 gm. of administered urea require some time to be absorbed. But this is not a valid explanation, for we know that at the very commencement of this period the blood urea concentration

[13] D'Amato, L., and Fagella, V., *Z. klin. Med.*, 1911, lxxii, 474. Schlayer, C., *Verhandl. kong. inn. Med.*, 1912, 501. Mosenthal, H., and Schlayer, C., *Deutsch. Arch. klin. Med.*, 1913, cxl, 217. Lindemann, *Ergebn. Physiol.*, 1914, xix, 652.

TABLE VII.

The Relationship between the Average Amounts of Administered Urea Excreted During Successive 4 Hour Intervals after the Ingestion of 20 Gm. of Urea, and the Average Amounts of Administered Urea within the Body.

Day.	Period.	Administered urea excreted in 4 hour periods.	Administered urea in body at commencement of each period.	Percentage of the administered urea in the body which was excreted every 4 hours.
4th	8 a.m.–12 n.	4.82	20.00	24
	12 n.–4 p.m.	4.37	15.18	29
	4–8 p.m.	3.23	10.81	30
5th	8 a.m.–12 n.	5.64	23.02	24
	12 n.–4 p.m.	5.37	17.38	31
	4–8 p.m.	3.38	12.01	28
6th	8 a.m.–12 n.	6.01	24.22	25
	12 n.–4 p.m.	5.23	18.21	29
	4–8 p.m.	3.24	12.98	25

rises rapidly to a level above that existing during the remainder of the day, so that the mean blood urea concentration is higher for this period than for any other. One would therefore expect that the largest instead of the lowest proportion of the administered urea in the body would have been eliminated. This is a good example of the fact that there are conditions under which there is not always a direct relationship between the concentration of urea in the blood and the rate of urea excretion.

The Rate of Excretion of Administered Urea after the Ingestion of 40 Gm. of Urea.

After the above data on the rate of urea excretion after the ingestion of 20 gm. of urea had been accumulated, we proceeded to determine the effect of the administration of double that amount of urea.

Nine subjects took 40 gm. of urea under the same conditions as before, except that the subjects K_4, Bo_3, Br, and Ca took the urea dissolved in 720 cc. of water instead of 360 cc. Unfortunately 4 hour collections were not made during the fore-period, so that we can only give the rate of excretion of administered urea for 24 hour and 12 hour periods. The results are given in Table VIII.

TABLE VIII.

Total urea excreted (fore-period).				Administered urea excreted after the ingestion of 40 gm. of urea.															
Average of 2nd and 3rd days.				4th day.				5th day.				6th day.				Average of 4th, 5th, and 6th days.			
24 hrs. 8 a.m.-8 a.m.		12 hrs. 8 a.m.-8 p.m.		24 hrs. 8 a.m.-8 a.m.		12 hrs. 8 a.m.-8 p.m.		24 hrs. 8 a.m. 8 a.m.		12 hrs. 8 a.m.-8 p.m.		24 hrs. 8 a.m.-8 a.m.		12 hrs. 8 a.m.-8 p.m.		24 hrs. 8 a.m.-8 a.m.		12 hrs. 8 a.m.-8 p.m.	
Subject	Urea	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.	Subject.	Urea.
	gm.		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*
Bo_3	19.18	T_2	11.03	Ca	38.16	Pr	29.42	Lo	42.74	St_2	32.40	Pr	47.10	Lo	32.85	Pr	40.66	Pr	30.60
T_2	18.83	Bo_3	10.00	Pr	37.64	Ca	28.74	St_2	41.79	Lo	31.65	Lo	45.38	St_2	32.52	Lo	40.44	Lo	29.87
K_4	17.25	Lo	9.75	Bo_3	36.62	Bo_3	28.28	T_2	40.33	Pr	31.44	T_2	42.97	T_2	31.81	T_2	39.21	T_2	29.57
Ca	17.04	Ca	9.66	Ba	36.05	Ba	28.07	Ba	38.33	T_2	30.73	St_2	41.43	Sh_2	31.16	Ca	39.08	Ca	29.22
St_2	15.57	K_4	9.21	Sh_2	35.15	Sh_2	27.68	Ca	37.44	Ba	29.39	Sh_2	40.79	Pr	30.96	Ba	38.01	Ba	28.71
Pr	15.42	Ba	8.65	T_2	34.34	T_2	26.17	Sh_2	37.31	Ca	28.26	Ca	40.66	Bo_3	30.74	Sh_2	37.75	Sh_2	28.48
Ba	14.59	St_2	8.58	K_4	33.51	Lo	25.13	Pr	37.26	K_4	28.03	K_4	39.63	Ca	30.66	K_4	36.36	Bo_3	28.38
Lo	14.50	Pr	8.52	Lo	33.22	K_4	24.39	K_4	35.95	Sh_2	26.60	Ba	39.63	K_4	30.63	Bo_3	35.80	K_4	27.68
Sh_2	13.57	Sh_2	7.12					Bo_3	32.90	Bo_3	26.12	Bo_3	37.88	Ba	28.89				
Average..	16.22		9.17		35.59		27.21		38.23		29.40		41.72		31.14		38.41		29.06
Average deviation.....					1.53		1.50		2.28		1.91		2.29		0.84		1.43		0.76

The average deviations show that the rate of excretion of administered urea is quite as constant after 40 as after 20 gm.

The average percentage of the 24 hour excretion of administered urea eliminated during the day and night periods was 75.6 per cent for the day time and 24.4 per cent for the night, as compared with 75.5 and 24.5 per cent after the ingestion of 20 gm. of urea. The ranges of variation were from 78 to 74 per cent for the day, and from 26 to 22 per cent for the night.

The relationship between the rate of excretion of administered urea and the amount of administered urea within the body is also substantially the same. After 40 gm. of urea the percentage of the administered urea within the body which was excreted during the 12 hours of the day was 64 per cent, whereas after 20 gm. of urea it was 61 per cent.

At the completion of the urea period an average of 4.46 gm. of the administered urea remained within the body when 40 gm. of urea were taken. The average amount after 20 gm. was 4.96 gm. The retention of this urea is not to be ascribed to an inability on the part of the kidneys to excrete it. It is retained because it has never been brought to the kidneys for excretion.

It would be useless to attempt by giving larger doses of urea to reach any absolute limit to the capacity of the normal kidney in excreting urea since no such limit exists for any dose of urea that it is possible to give. As long as sufficient water is available the rate of excretion will increase with every increase in the amount of urea ingested.

CONCLUSIONS.

1. In thirty-nine experiments on young healthy adults no evidence of variability in kidney action could be obtained in the work of excreting preformed urea added to a constant diet. Such variation as was found in the amounts of urea excreted could be fully accounted for as arising from extra-renal factors. It is concluded therefore that the normal kidney under constant conditions is characterized by the possession of a high degree of constancy of function.

2. The rate of excretion of the administered urea during successive periods of the 24 hours showed that the repetition of

large doses of urea did not elicit the condition which has been described as "kidney fatigue."

3. A fraction of the urea which was administered remained in the body after 24 hours. This urea was not retained because of any failure on the part of the kidneys to eliminate it, for the amount retained was no larger when 40 than when 20 gm. of urea were taken.

THE VOLUME OF URINE IN YOUNG HEALTHY ADULTS ON A CONSTANT DIET.

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In the course of an investigation into the conditions controlling the rate of urea excretion, we have accumulated data in regard to the volume of urine of subjects on a constant water intake, which are of themselves of interest as illustrating the wide range of variation of this factor, and may further prove of value in defining the limits beyond which a given volume of urine may properly be regarded as abnormally large or small.

The diet has been detailed in the previous paper on the rate of urea excretion. The total water content of the food and fluids was 1,710 cc. This was increased to 2,070 cc. on the last 3 days of the diet by the 360 cc. of water in which the urea taken was dissolved. A slight inequality in water intake was introduced in those few cases who did not always take all of the nitrogen-free cornstarch which was provided.

Table I gives the averages and limits of variation in the volume of urine in those twenty subjects who took 20 gm. of urea under the above conditions.

It is evident from these figures that the volume of urine in any individual for any one day or part of a day is an extremely variable quantity, in spite of constant water intake. But when the volumes of the 4th, 5th, and 6th days in each individual are averaged, the range of variation is greatly lessened. These averages are given in order of ascending magnitude in Table II.

We hope to find the above figures useful in the recognition of true polyuria or oliguria.

When the kidneys are diseased, the volume of urine passed at night is often larger than the day urine. But we have found

that this condition may be present in subjects whose kidneys are perfectly normal. When, however, the percentages of the total 24 hour urine excreted at night on the 4th, 5th, and 6th days are averaged for each individual, we find that in all of them the night excretion is less than the day. Table III shows the average and limits of variation in the average percentage distribution of urine volume for 3 days.

TABLE I.

Average and Limits of Variation in Volume of Urine (Cc.).

Period.	1st day.			2nd day.			3rd day.		
	Average.	Limits of variation.		Average.	Limits of variation.		Average.	Limits of variation.	
		Highest.	Lowest.		Highest.	Lowest.		Highest.	Lowest.
24 hrs., 8 a.m.–8 a.m.	1,058	2,215	609	1,059	2,023	727	975	1,341	587
12 hrs., 8 a.m.–8 p.m.	642	1,405	381	646	1,272	366	617	862	395
8 hrs., 8 a.m.–4 p.m.	458	1,141	281	413	581	225	417	646	284
	4th day.			5th day.			6th day.		
24 hrs. 8 a.m.–8 a.m.	1,406	2,323	951	1,354	1,674	1,003	1,322	1,665	903
12 hrs., 8 a.m.–8 p.m.	902	1,681	605	913	1,248	732	893	1,269	615
8 hrs., 8 a.m.–4 p.m.	643	1,149	422	661	925	446	645	1,035	477

There is not less than 38 per cent of the 24 hour volume excreted during the first 8 hours of the day, and not less than 53 per cent in the first 12. Under these conditions also we never find more than 47 per cent eliminated during the night.

We may therefore be reasonably certain of the presence of a true nocturia, meaning an abnormally high proportion of night urine, when we find in any individual who takes the diet that 50 per cent or more of the average 24 hour excretion of the last 3 days is eliminated at night.

TABLE II.

The Average Volume of Urine of the 4th, 5th, and 6th Days.

No.	8 hrs. (8 a.m.—4 p.m.)		12 hrs. (8 a.m.—8 p.m.)		24 hrs. (8 a.m.—8 a.m.)	
	Subject.	Volume.	Subject.	Volume.	Subject.	Volume.
		cc.		*cc.*		*cc.*
1	Br_1	501	A_1	684	A_1	1,013
2	A_1	519	Br_1	709	Br_1	1,022
3	Ad	519	Ad	713	Ad	1,097
4	Bo_1	527	Bo_1	740	S_1	1,202
5	G	528	D	813	Bo_1	1,213
6	D	603	G	819	F	1,234
7	J	604	K_2	833	We	1,253
8	P	613	F	868	D	1,257
9	F	626	P	879	P	1,297
10	K_2	626	J	901	J	1,345
11	Cr	691	K_1	901	G	1,378
12	K_1	700	We	924	Cr	1,393
13	We	707	Boy_1	935	Boy_1	1,399
14	B	708	B	964	S_2	1,441
15	S_1	724	K_3	968	M	1 441
16	S_2	734	Cr	1,011	B	1,467
17	K_3	734	S_2	1,017	K_3	1,489
18	Boy_1	765	S_1	1,042	K_2	1,526
19	M	773	M	1,063	Je	1,707
20	Je	788	Je	1,195	K_1	1,712
Average.		650		903		1,361

TABLE III.

Average and Limits of Variation in the Average Percentages of Urine Volume Excreted During Different Periods of the 24 Hours on the 4th, 5th, and 6th Days.

Period.	Average.	Highest.	Lowest.
	per cent	*per cent*	*per cent*
8 hrs. of day, 8 a.m.–4 p.m.	48	56	38
12 " " " 8 a.m.–8 p.m.	66	74	53
12 " " night, 8 p.m.–8 a.m.	34	47	26

In order to determine whether a moderate increase in the volume of urine led to any increase in the rate of excretion of administered urea, the experiment was repeated in four subjects under

the same conditions except that 250 cc. of water were taken with each of the four meals, in all 1,000 cc. of additional water for each 24 hour period. Table IV gives the average amounts of administered urea excreted by these four individuals with and without extra water.

TABLE IV.

No Extra Water.

Period.	Average amounts of administered urea excreted.				Volume of urine.
	4th day.	5th day.	6th day.	Average of 4th, 5th, and 6th days.	Average of 4th, 5th, and 6th days.
					cc.
24 hrs., 8 a.m.–8 a.m.	15.3	20.0	19.9	18.4	1,128
12 " 8 a.m.–8 p.m.*	11.3	15.7	14.1	13.7	752
8 " 8 a.m.–4 p.m.**	9.3	11.5	10.6	10.5	545
1,000 Cc. Extra Water Per Day.					
24 hrs., 8 a.m.–8 a.m.	17.0	19.4	20.4	18.9	2,350
12 " 8 a.m.–8 p.m.*	12.2	13.7	16.3	14.1	1,671
8 " 8 a.m.–4 p.m.**	10.2	12.2	13.2	11.9	1,277

*Average of three cases only.
** " " two " "

While the figures for the 4th and 6th days suggest a slight acceleration of the rate of excretion, yet the averages for the 3 days are almost identical. These differences are well within the limits of error, and it therefore cannot be said that there is any demonstrable increase in the rate of excretion of administered urea when 1,000 cc. of water a day are added to the diet. We do not conclude that the volume of urine never has any effect on the rate of urea excretion. On the contrary, we have evidence which will be given later that under certain well defined conditions the volume of urine is a very important factor in determining the rate of excretion. But under the above conditions doubling the volume of urine has no appreciable influence.

It may be worth noting that there is no indication that the extra water altered the level of protein catabolism. The average total excretion of urea for the whole period of 6 days was 152 gm.

when the total urine volume was 6,484 cc., and 149 gm. when the volume was 12,827 cc.

Although the rate of urea excretion is not demonstrably increased by an increase in urine volume, an increase in the rate of urea excretion induced by the ingestion of urea is accompanied by an increased urine volume. The average volumes of urine excreted on the 4th, 5th, and 6th days of the diet by three groups of individuals who took different amounts of urea are compared in Table V. The first group consists of six individuals who were given 360 cc. of water but no urea, the second comprises twenty who took 20 gm. of urea in 360 cc. of water, and the third group is compiled from five individuals who were given 40 gm. of urea in 360 cc. of water.

TABLE V.

The Average Volume of Urine of the 4th, 5th, and 6th Days in Groups of Individuals Who Took No Urea, 20 Gm. of Urea, or 40 Gm. of Urea.

Period.	No urea.	20 gm. of urea.	40 gm. of urea.
	cc.	cc.	cc.
24 hrs., 8 a.m.–8 a.m.	1,051	1,361	1,446
12 " 8 a.m.–8 p.m.	707	903	988
8 " 8 a.m.–4 p.m.	547	650	735
4 " 8 a.m.–12 noon	245	337	398

In spite of the constancy of the water intake the average volume of urine is considerably increased in those who took urea.

CONCLUSIONS.

1. The volume of urine in normal individuals on a constant diet with the same water intake is extremely variable for any single day or part of a day.

2. The average volume of the last 3 days of the diet when the water intake was 2,070 cc. varied in twenty individuals from 1,013 to 1,712 cc. for a 24 hour period, from 684 to 1,195 cc. for the first 12 hours of the day, and from 501 to 788 cc. for the first 8 hours of the day.

3. The percentage of the 24 hour volume excreted during the 12 hours of the night did not exceed 47 per cent in any subject, when the volumes for the last 3 days of the diet were averaged.

4. An increase of 1,000 cc. a day in the water intake more than doubled the volume of urine but did not appreciably increase the rate of urea excretion.

5. An increase in the rate of urea excretion induced by the ingestion of urea, the water intake remaining constant, was accompanied by a considerable increase in the volume of urine.

OBSERVATIONS ON THE CHANGES IN THE CHOLESTEROL CONTENT OF THE BLOOD OF GOATS, FOLLOWING CHOLESTEROL FEEDING ALONE, ROENTGEN TREATMENT ALONE, AND CHOLESTEROL FEEDING COMBINED WITH ROENTGEN TREATMENT AND SUBSEQUENT CASTRATION.*

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The importance of cholesterol metabolism has been established by the work of Weltmann (1), Bacmeister and Havers (2), Aschoff (3), McNee (4), Wacker (5), Sternberg (6), Rothschild (7), and others; that its study is becoming a subject of general interest may be seen from the number of observations that have been made within the last 2 years.

While the main object of the earlier work done on cholesterol was the experimental reproduction of the arteriosclerotic lesions (Stuckey (8), Wesselkin (9), Chatalow (10), Anitschkow (11), and lately McMeans (12)), this work proved incidentally that a great number of organs are affected by a disturbance of the cholesterol balance. Subsequent investigations have shown that the organs principally concerned with the regulation of the cholesterol metabolism are the adrenals, the liver, the genital glands, and the intestines (Rothschild (7), Weltmann and Biach (13), Anitschkow and Chatalow (14), Albrecht and Weltmann (15), Stewart (16), Hueck (17), Gardner and Lander (18), Sternberg (6), Lowenthal, (19), McMeans (12), McNee (4)). That cholesterol is also stored in the body fat has been demonstrated by Rothschild (20) and Wacker (5). It has further been shown that the cholesterol content of the blood is changed by physiologic as well as by pathologic conditions; that pregnancy and lactation, diseases of the liver, and malignant growths increase cholesterol value, but that the influence of bacterial infection depends on the acute or chronic character of the process; Weltmann (1) found that in tuberculosis, for instance, the cholesterol content of the blood was lowered as the disease progressed.

The effect of cholesterol on cell proliferation has been made a subject of recent investigations: Robertson and Burnett (21) reported that a growth of transplanted carcinoma in white rats (Flexner-Jobling type)

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was accelerated by the intravenous injections of cholesterol emulsion; Browder (22) states that the rate of cell division of paramecia is markedly increased by the addition of small amounts of cholesterol to the culture medium; McMeans (12) observed that proliferation of the arterioles of the lungs and the kidneys occurred in cholesterol-fed rabbits. The bearing of these experiments on the problem of malignant growth is obvious.

The foregoing brief review shows that a great number of factors influence cholesterol metabolism; *viz.*, the relative adequacy of some four or five organs taking part in the regulation of the metabolic balance and physiologic as well as pathologic conditions. There may be other factors whose importance has not yet been ascertained, and it will therefore be easily understood that the interpretation of changes in the cholesterol value of the blood is fraught with difficulties. At present only deductions based on unusually high, or unusually low, on markedly progressive, or markedly recurrent cholesterol values can be expected to give any definite clue concerning the actual relation between a disturbance of the cholesterol balance and the associated pathologic symptoms.

The object of my experimental work is to produce malignant proliferation in animals by disturbing the cholesterol balance. For this purpose cholesterol feeding has been used. In some cases Roentgen treatment has been employed in addition, to break down the "lymphoid defence" of the animal (Murphy and Morton (23)). Cholesterol tests of the blood were made, not as an end in themselves, but as a means by which the progress of the metabolic disturbance might be gauged. A few observations made during the course of the experiment seem to furnish data concerning some of the factors that influence the cholesterol content of the blood. These observations I wish to present here although the experiment under discussion is not yet completed.

Rothschild (7) in his studies on the cholesterol content of the blood of rabbits found that normal animals appear to have an "individual cholesterol standard" to which they adhere with only slight variations. Bloor (24) has observed that in dogs the process of digestion and the chemical constituents of certain types of food do not influence the cholesterol percentage to any considerable extent. In the main, this may also be applied to goats (Table I, Fig. 1).

TABLE I.

Cholesterol Values in the Blood of Normal Goats. Tests Made Every 2 Hours (8 a.m. to 6 p.m.). Results Expressed in Per Cent.

Time.	Readings.*	Highest value.
	Goat 21 (male; received cholesterol only).	
8 a.m.	0.216 - - 0.216 - - 0.272** - - - (0.216)†	0.272
10 "	0.186 - - 0.216 - - 0.254 - - 0.254 - - 0.266** - - - - - (0.230)	0.266
12 m.	0.242 - - 0.242 - - 0.262 - - 0.262 - - 0.266** - - - - - - (0.216)	0.266
2 p.m.	0.254 - - 0.254 - - 0.266** - - - - - - (0.224)	0.266
4 "	0.216 - - 0.216 - - 0.242 - - 0.242 - - 0.272** - - - - (0.242)	0.272
6 "	0.210 - - 0.210 - - 0.242 - - 0.242 - - 0.260** - - - - (0.220)	0.260
	Goat 10 (male; normal control).	
8 a.m.	0.158 - - 0.158 - - 0.180 - - 0.180 - - 0.202** - - - - - - (0.168)	0.202
10 "	0.146 - - 0.146 - - 0.186** - - - - - - - (0.158)	0.186
12 m.	0.158 - - 0.158 - - 0.186** - - - - - - - - (0.158)	0.186
2 p.m.	0.168 - - 0.168 - - 0.190 - - 0.190 - - 0.202** - - - - - - (0.158)	0.202
4 "	0.186 - - 0.186 - - 0.202** - - - - - - (0.168)	0.202
6 " ‡	0.130 - - 0.130 - - 0.186** - - - - - - - (0.146)	0.186

*The readings made at each 2 hour period were repeated at intervals of 2 minutes.

** Value remained constant for four or more readings.

† The small dashes represent approximately the number of minutes that elapsed between the last constant reading and the first reading lower. Note that this interval was longer for Goat 10 than for Goat 21.

‡ The slightly lower values at this time may have been due to the fact that a few clots had formed.

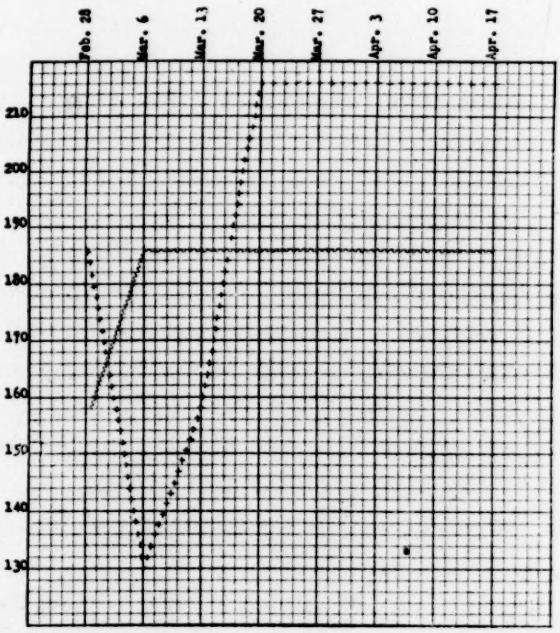

FIG. 1. Cholesterol values in the blood of two normal goats; Goat 10, male, ~~~~~~~; Goat 35, female, +++. Note that the values for Goat 10 remained constant for 7 weeks and those for Goat 35 remained constant for 5 weeks.

Fig. 1 shows the cholesterol values found in the blood of two normal goats, one male (G10) and one female (G35), during a period of 8 weeks (weekly tests). Whereas the values of the male animal remained unchanged for 7 consecutive weeks, a drop occurred in the cholesterol value of the female between March 6 and 13, the animal being in heat at the time. The fact that a lower cholesterol value was observed while the animal was in heat indicates that the cholesterol content of the blood is influenced by reproductive activity, an observation to be discussed at greater length in connection with the period following castration.

Table I gives the cholesterol values found by tests made every 2 hours from 8 a.m. to 6 p.m. The effect of the various stages of digestion even in a ruminating animal should be clearly demonstrable by an experiment extending over a period of 10 hours. Whether the slight variations that occurred are really due to the digestive process is a difficult matter to decide, since a goat ruminates and digests without interruption so that even if food were kept out of its reach for some time, the digestive factor could not be entirely eliminated. (Starvation increases the cholesterol content, as Rothschild has ascertained. However, it would seem practically impossible to regulate the food supply for a ruminating animal so that digestion would cease without risking the occurrence of slight symptoms of starvation.) Whether the fluctuations of the cholesterol content (Table I) are due to the process of digestion or to a greater amount of muscular activity on the part of the animal, they are in themselves so slight that they can hardly be considered significant.

Method of Investigation.—In my experiment, the cholesterol content of the blood of six goats was determined every week in the course of 7 months. For obvious reasons this part of the experiment is divided into three periods: (1) Observation and cholesterol feeding alone (Fig. 2). (2) Continued cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment (Figs. 3a, 3b, and 3c). (3) Following castration, other experimental factors unchanged (Figs. 5a, 5b, and 5c).

First Period.—During the first 5 weeks all the goats were kept under good laboratory conditions and were not subjected to any

experiment except that 3 cc. of blood were taken from the jugular vein by means of a hypodermic syringe every week in order to determine the average cholesterol value of the blood of each animal. At the end of 3 weeks all the animals developed symptoms of mange. The disease remained confined to small areas on the ears or nose and apparently cleared up in a fortnight. The changes occurring in the cholesterol value from Oct. 7 to Nov. 1 may be partly accounted for by this infection, and partly by the fact that the goats, having been on free range, had not yet become acclimated to laboratory conditions and stable food. On Nov. 1 two of the animals (G19 and G21) were fed daily 0.30 gm. of cholesterol in capsules, that the effect of this substance on the cholesterol content of the blood might be studied. The extraordinary digestive powers of the goat which cause this animal to thrive on the most indigestible articles seemed to justify the apprehension that the cholesterol might be eliminated without any apparent effect. However, as may be noted from Fig. 2, anxiety on this point proved without foundation, for whereas two of the controls (G16 and G26) did not make up the deficit in their cholesterol value, and the other two (G17 and G20) exceeded their original percentage by a small margin only (0.006–0.018), the two cholesterol-fed animals (G19 and G21) gained 0.90 per cent; that is, the cholesterol content of the blood increased by 0.090 and 0.062 mg. respectively, as compared with the original values. Therefore, from Fig. 2, the following conclusions may be drawn.

1. The cholesterol content can be increased in the blood of goats by cholesterol feeding.

2. Parasitic infection (mange) apparently decreases the cholesterol value of the blood. This deduction has been justified by later observations during a recurrence of mange after the animals had become thoroughly acclimated to laboratory conditions and were steadily putting on weight. The fact may perhaps be explained as the result of an effort on the part of the organism to combat the infection.

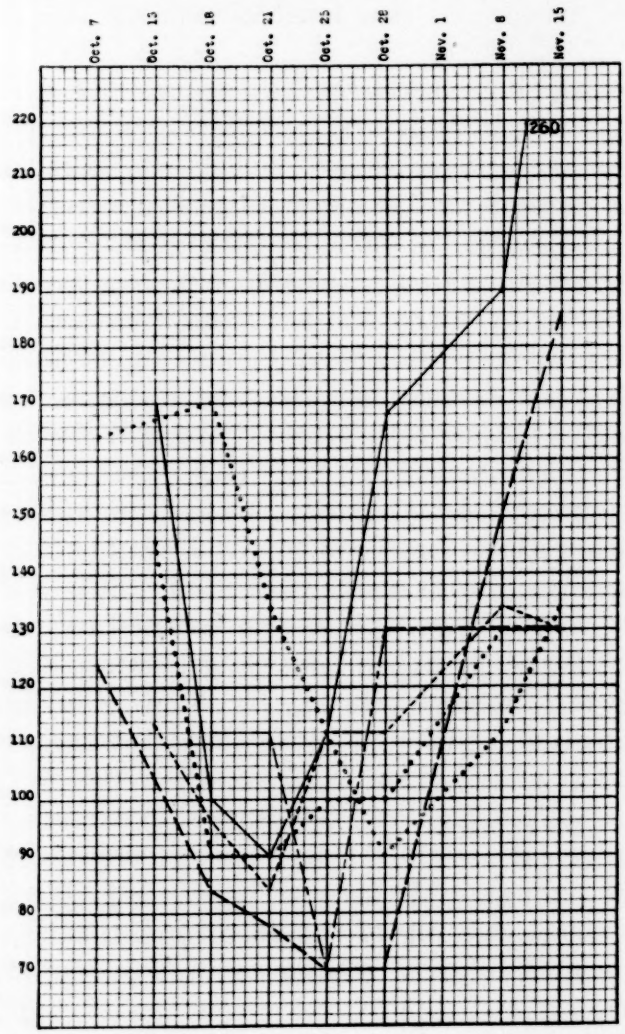

FIG. 2. Cholesterol values for Period I. Goat 16, ° ° °; Goat 17, — — —; Goat 19, ———; Goat 20, – – –; Goat 21, — — —; Goat 26, ...
Note the drop in the values for all of the goats during infection (mange) and the increase in the values for Goats 19 and 21 on cholesterol feeding.

Second Period.—During the second period observations were made on the effect of cholesterol feeding alone, of Roentgen treatment alone, and of Roentgen treatment combined with cholesterol feeding, the six goats being divided into three corresponding groups; *viz.*, G19 and G21 (Group A) continued to get 0.30 gm. of cholesterol daily in capsules; G16 and G26 (Group B) diffuse Roentgen treatment (diaphragm wide open) consisting of a 12.5 milliampere per minute dose daily for 7 days (2.5 milliamperes at a distance of 24 inches from the tube, exposure 5 minutes) followed by 7 days' rest; G17 and G20 (Group C) were given the same Roentgen treatment but received in addition 0.30 gm. of cholesterol daily without interruption (Figs. 3a, 3b, and 3c).

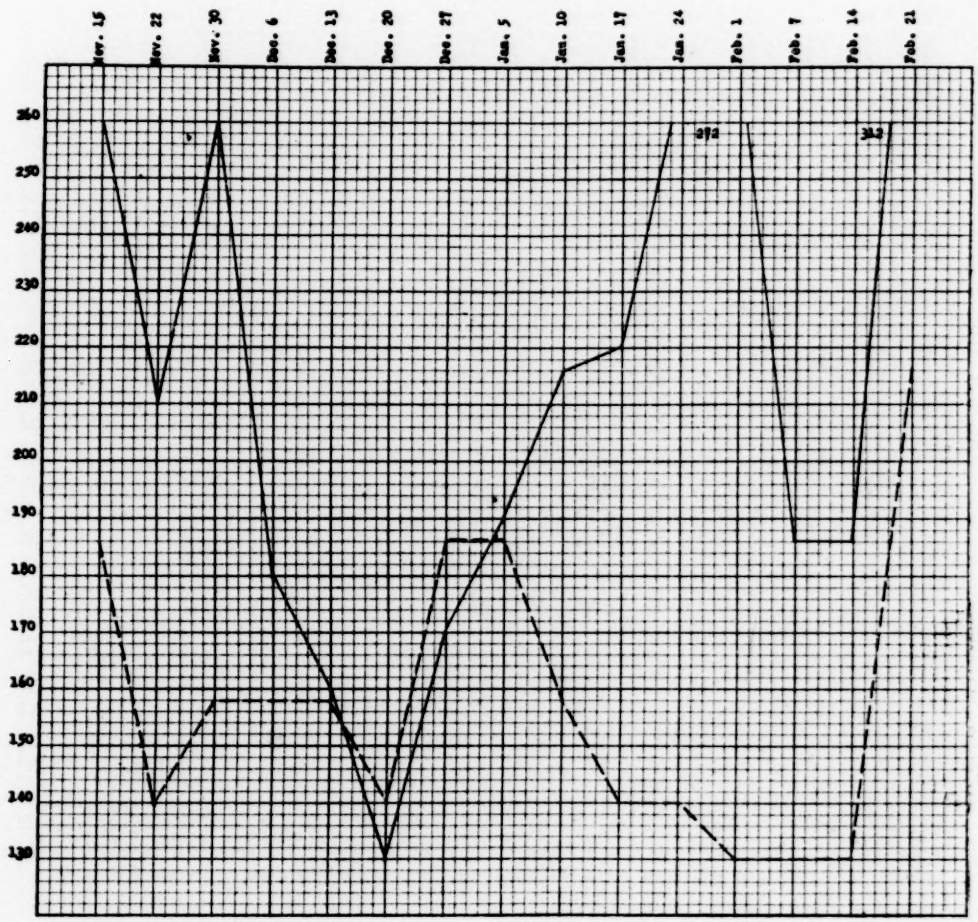

FIG. 3 a. Cholesterol values for Group A, Period II. The effect of cholesterol feeding and mange. Goat 19, ———; Goat 21, — — —.

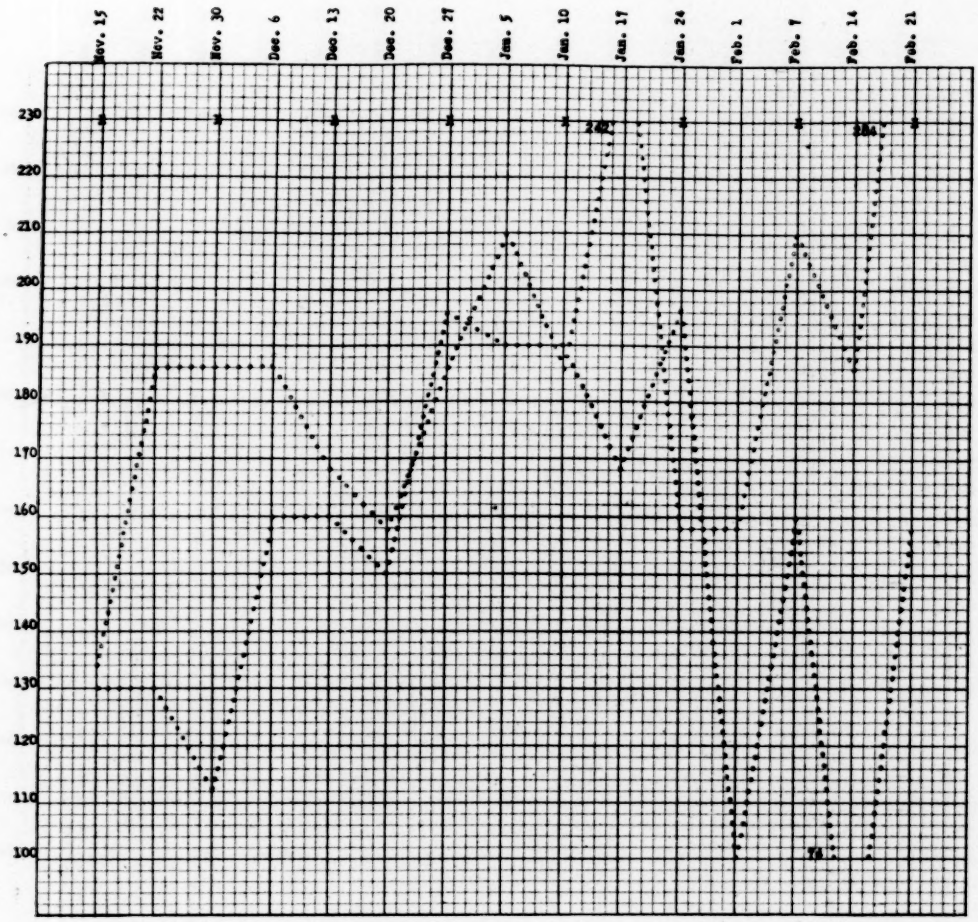

FIG. 3 b. Cholesterol values for Group B, Period II. The effect of Roentgen ray treatment and mange. Goat 16, ° ° °; Goat 26 ..... X indicates Roentgen ray treatment.

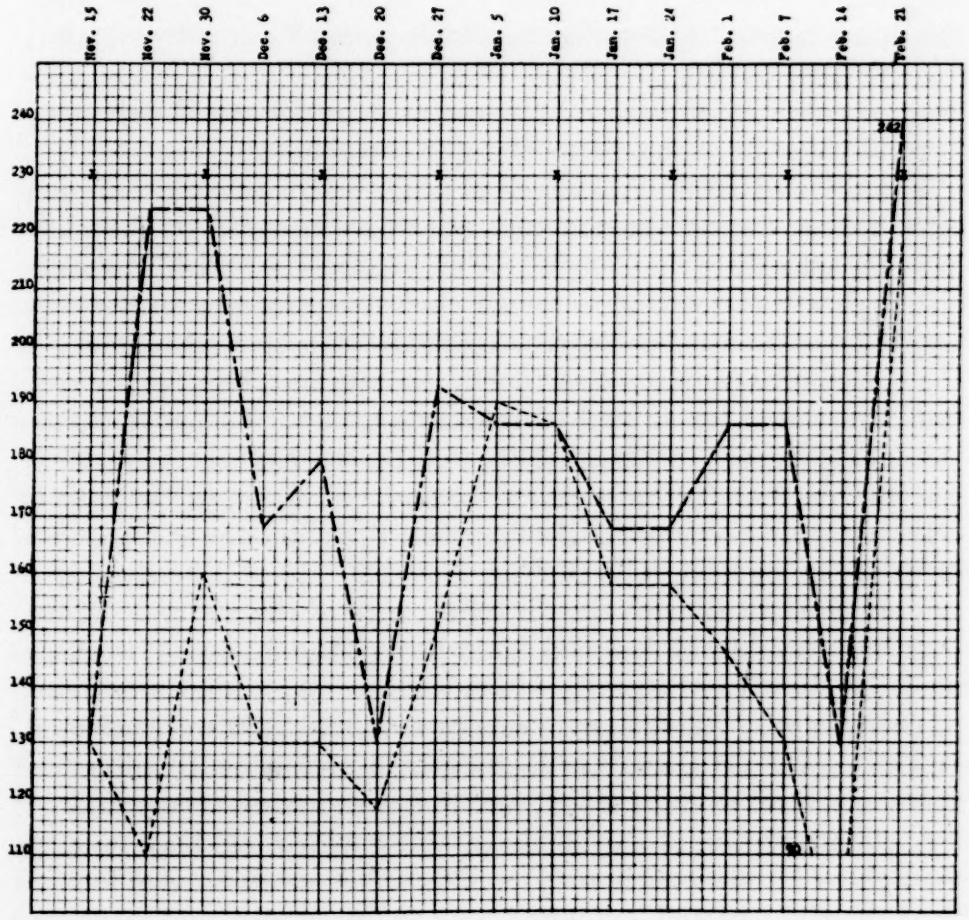

FIG. 3 c. Cholesterol values for Group C, Period II. The effect of Roentgen ray treatment and cholesterol feeding combined (in the values for Goat 17, the additional influence of pregnancy). Goat 17, —— - ——; Goat 28, — — —. X indicates Roentgen ray treatment.

The interpretation of the results obtained during this period is extremely difficult owing to the number of intercurrent factors, the effect of which on the cholesterol content of the blood can be merely guessed, since at present data for comparison are not available and can be obtained only by further investigations. On the other hand, the conclusion which seemed warranted by my observations during the first period, *viz.*, the increase of the cholesterol value by cholesterol feeding and the decrease of the cholesterol value by mange, appear to be corroborated.

Thus, G19 (Group A), which already had the highest cholesterol values during the previous period, will again be found to have higher values than any of the other animals, and G21, which, although it had responded to the cholesterol feeding previously, had done so to a lesser degree, again shows a general increase in the cholesterol content of its blood, less marked than the increase found in the other animal of the same group. Closer examination of the curves in Figs. 3a, 3b, and 3c reveals the influence of the intercurrent factors referred to above. Thus, in the case of G19, a severe recurrence of mange on the shoulder explains the drop of the cholesterol value on Nov. 22. It will be seen that the value went up with the disappearance of the mange. Moreover, the cholesterol dropped again during the following weeks, a decrease that may be partly accounted for by another recurrence of the mange. All the goats except G16 and G20 were affected. In the case of G19, another factor which undoubtedly influenced the cholesterol content from Dec. 13 onward must be considered; *viz.*, the formation of a cyst-like swelling which had become large enough to be noticeable on Dec. 30. This swelling was located near the right nostril, extended over the upper lip, and measured about 2 cm. in diameter. Slight fluctuation was present at first, suggesting that it might be an abscess. The fluctuation then disappeared, and the swelling assumed a tumor-like aspect. To ascertain the exact nature of the tumor, it was lanced on Jan. 17, and found to contain pus. A small quantity (1.2 gm.) of the pus was taken for chemical analysis, and the remainder left to be reabsorbed by the animal. A test of the pus for cholesterol showed the cholesterol value to be ten times that of the blood and seemed to warrant the assumption that the abscess was a "deposit" for the increasing surplus cholesterol in the blood. This supposition

appeared to be corroborated by the increase of the cholesterol value in the blood during the following weeks, when the remainder of the abscess was being slowly reabsorbed. Several tests made with pus from patients did not give any higher cholesterol values than are usually found in the blood under conditions of slight inflammation. The fluctuations in the cholesterol value of the blood of G21 would appear to be due to the appearance and disappearance of mange.

For the changes in the cholesterol values observed in all of the animals between Feb. 1 and 21, no apparent reason can be found unless extreme changes in the temperature (days on which the thermometer registered 25–40° below zero, alternating with days of warm spring-like weather) are to be taken into account.[1] It is quite conceivable that similar extremes of heat and cold cannot be without effect on general metabolism.

The result of the Roentgen treatment (Group B) is not sufficiently striking to permit of any definite conclusions. The fact that a slight drop in the cholesterol value occurs in several instances toward the end of the second period after a week of Roentgen treatment (Jan. 24 to Feb. 21, G16, G17, G20, and G26), and is followed by a slight increase after a week of rest, suggests the possibility that the Roentgen ray may have a depressing effect on the cholesterol value in the blood. Similar changes were found to occur during the third period. The present data are still too meager to furnish any conclusive evidence although they appear to be in accordance with Soper's (25) observations on the effect of mesothorium rays on the cholesterol value in the blood of rabbits. Soper reports a decrease of the cholesterol content in his animals after mesothorium treatment, but considers it too slight to be significant since it was not corroborated by histologic changes in the spleen. The short duration of his experiment (9 to 11 days) and the fact that several organs play a part in the regulation of cholesterol metabolism might explain his results. If, however, the reduction of the cholesterol content by Roentgen rays should become more marked in the course of my experiments or be confirmed by independent studies, it might furnish valuable

[1] Though the animals were kept in stalls, warmed by the central heating system, there was an outside run in which they spent a great part of the day, apparently by preference.

information concerning the beneficial effect of the Roentgen rays on malignant conditions, since the stimulating effect of cholesterol on cell proliferation demonstrated experimentally, and the high cholesterol values found in the blood of a number of cancer patients, seem to indicate that there may be a close connection between disturbances of cholesterol metabolism and malignant growth.

The possible effect of extreme and sudden changes of temperature on the cholesterol content of the blood has been mentioned in the discussion of Group A.

In Group B, the influence of mange on the cholesterol values is but faintly indicated. G26 alone had a slight recurrence, and it is difficult to decide whether the drop from 0.160 to 0.150 per cent (Dec. 13 to 20) is to be attributed to the disease or to the Roentgen treatment. On Jan. 10 G16 developed about half a dozen small abscesses in the inguinal region, none of which were larger than a pea. These abscesses cleared up without treatment and on Feb. 7 had completely disappeared. As the postules were too small to permit the taking of any pus for chemical analysis, it is impossible to say whether their reabsorption could have caused the changes found in the cholesterol value on Jan. 10 and 17, or were in any way comparable to the abscess on the nose of G19.

By combining Roentgen treatment with cholesterol feeding (Group C) I hoped to ascertain which of the two forms of treatment exerted the greater influence on the cholesterol content of the blood. The time allowed for this part of the experiment, 4 months, seemed amply sufficient to warrant definite results. However, the cholesterol values registered do not furnish conclusive data, although they suggest that Roentgen treatment and cholesterol feeding may have neutralized each other; subsequent investigations will have to settle this point. In regard to parasitic infection, a slight recurrence of mange (G17, Dec. 13 to 20) appears to corroborate my previous findings, but another factor will also have to be considered, *viz.*, the influence of pregnancy in combination with Roentgen treatment and cholesterol feeding. This will be discussed briefly in connection with the third period.

Summarizing briefly the foregoing:

1. The increase in the cholesterol value of the blood resulting

from cholesterol feeding and the decrease resulting from mange observed in the first period has been confirmed by the findings in the second period.

2. The effect of the Roentgen rays cannot yet be determined definitely owing to the influence of intercurrent factors; the results suggest the possibility of a decrease of cholesterol values in the blood due to Roentgen treatment.

3. The effect of combined Roentgen treatment and cholesterol feeding suggests that these factors may neutralize each other, but the influence of intercurrent factors makes definite conclusions premature, and emphasizes the need of further investigations.

Third Period.—During this period the effect of castration was studied as an additional factor. The measure was suggested by the influence on malignant conditions of the "cancer age," *viz.*, the age at which reproductive activity decreases. It will be remembered that the principal aim of the experiment was the reproduction of malignant proliferation. One goat of each one of the three groups referred to in the discussion of the second period was castrated. Thus, in Group A (cholesterol feeding alone) G19 was castrated, and both goats belonging to this group continued to be fed 0.30 gm. of cholesterol daily; in Group B G16 was castrated, and both animals belonging to this group (G16 and G26) continued to be treated with the same doses of Roentgen rays; in Group C the male (G20) was castrated, and the combina ion of Roentgen treatment and cho esterol feeding was continued as before.

In order to verify previous observations on the changes in the cholesterol content caused by anesthesia, samples of the blood were taken from 10 to 15 minutes after the goats had been given $\frac{1}{4}$ grain of morphine, before the administration of the anesthetic, and again on completion of the operation when the animal was still in narcosis. In every instance there was a decrease in the cholesterol content below the value obtained before the anesthetic was given and similar to the decrease observed in dogs (Fig. 4). An additional test was made with the blood of G16, taken 5 minutes after the beginning of anesthesia (the animal being completely unconscious) and demonstrated the fact, observed also in experiments on dogs, that anesthesia is at first accompanied by an increase of the cholesterol content, but that lower values

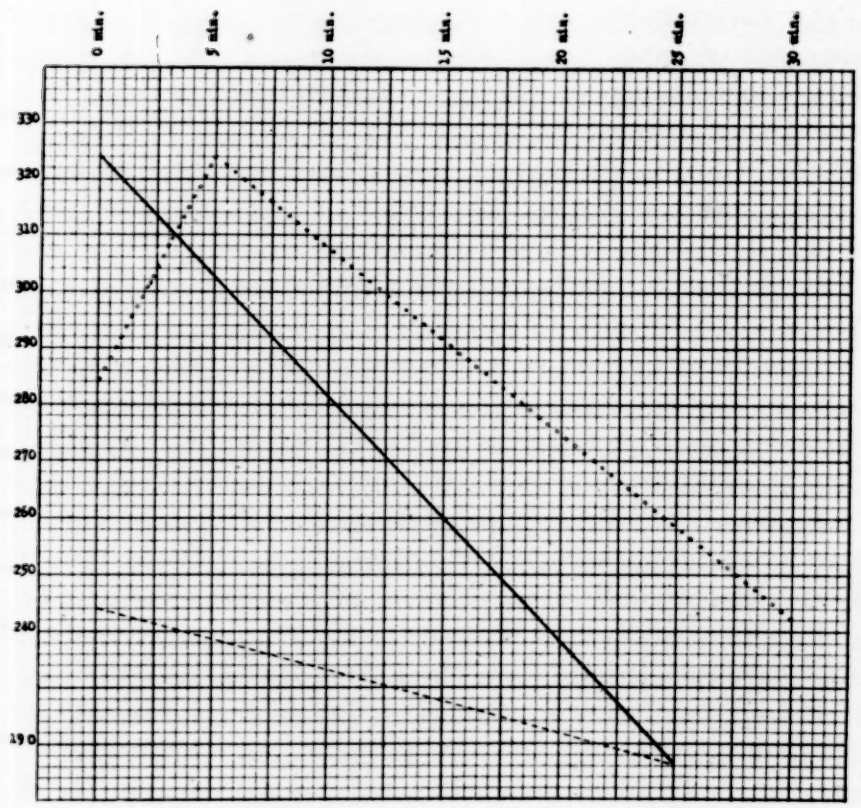

FIG. 4. The effect of anesthesia on cholesterol values. Goat 16, ° ° °; Goat 19, ———; Goat 20, — — —.

occur after the anesthetic has been given for some time. The length of this period appears to vary in different animals. Experiments on the effect of anesthesia on the cholesterol content of the blood are still in progress. The influence of the operative procedure itself need not be taken into consideration, since in our experiments on dogs the above results were obtained by means of the anesthetic alone.

The effect of castration is illustrated in Figs. 5a, 5b, and 5c. In Groups A and B increase of the cholesterol content following castration may be observed by comparing the values of the castrated and the non-castrated animal. In Group C the difference is less marked as the intercurrent factor of pregnancy in the animal intended for a control (G17) is likely to have increased the cholesterol values.

During the third period (Figs. 5a, 5b, and 5c) the highest cholesterol values of all were found in the blood of G19 (cholesterol-fed and castrated), the lowest values were found in the blood of G26 (no castration, Roentgen treatment only), while intermediate values could be observed in the blood of G16 (Roentgen treatment and castration).

It is further interesting to note that although the cholesterol value of G19 dropped as low as that of G20 (0.186 per cent) after anesthesia, the animal receiving cholesterol only went up to 0.330 per cent during the week following operation, whereas G20 which received cholesterol in combination with Roentgen treatment went down to 0.130 per cent.

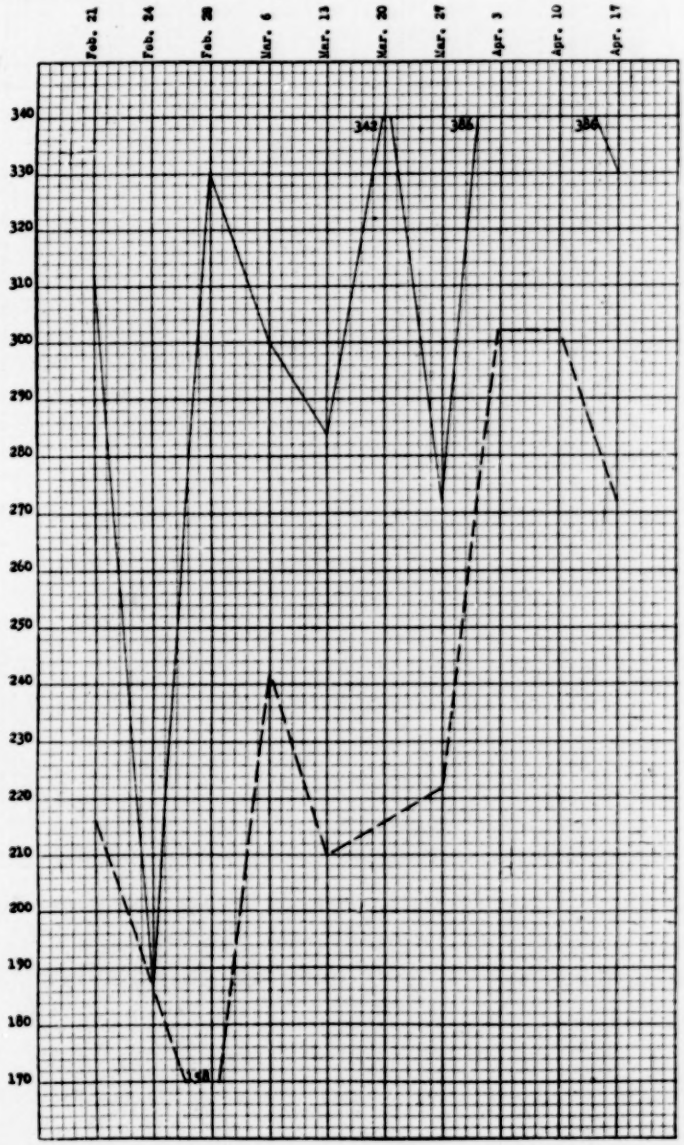

FIG. 5 a. Cholesterol values for Group A, Period III. The effect of cholesterol feeding (Goat 21, — — —) and of cholesterol feeding and castration (Goat 19, ———).

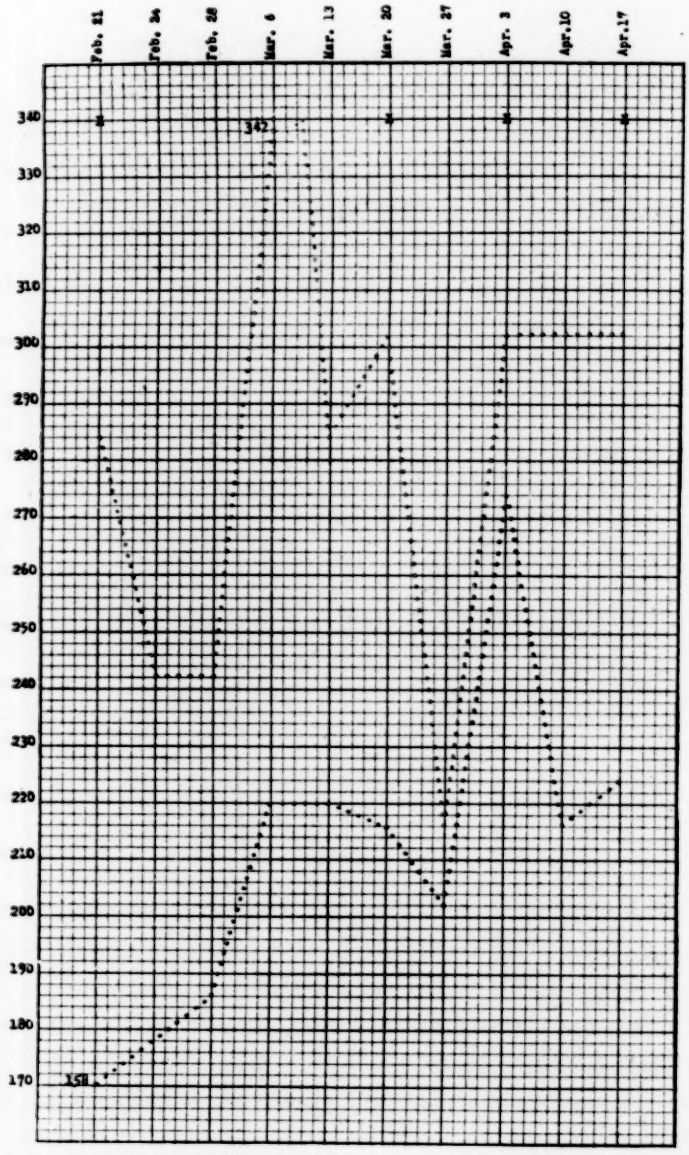

FIG. 5 b. Cholesterol values for Group B, Period III. The effect of Roentgen ray treatment (Goat 26, ...) and of Roentgen ray treatment and castration (Goat 16, ° ° °). X indicates Roentgen ray treatment.

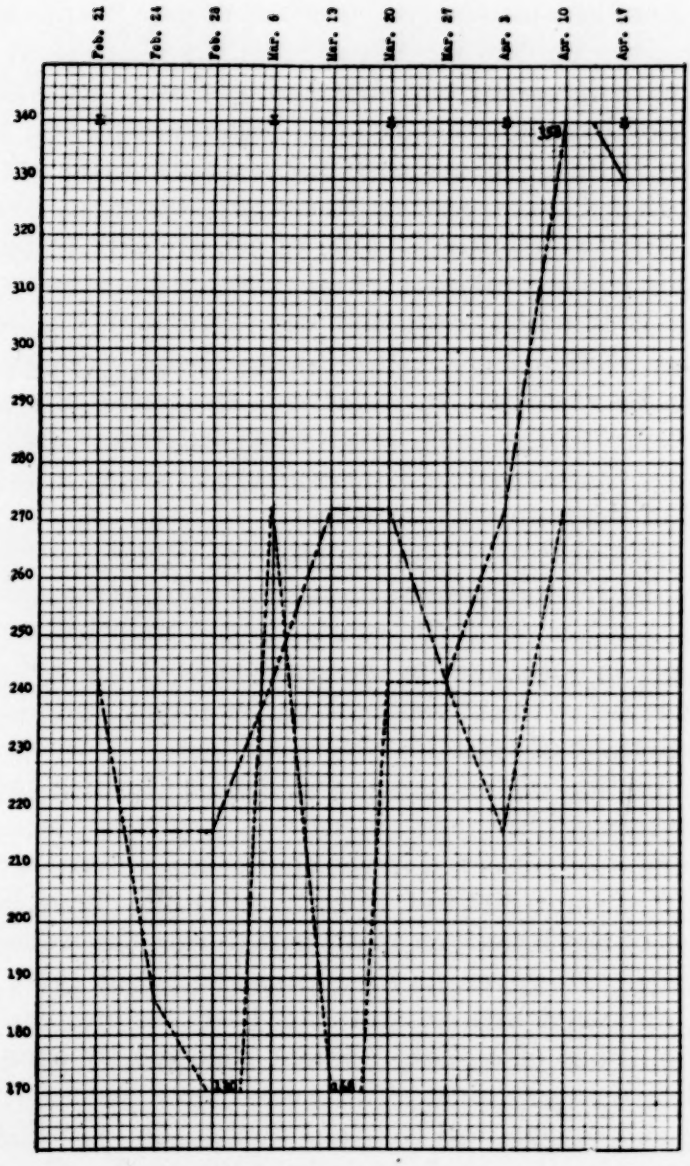

FIG. 5 c. Cholesterol values for Group C, Period III. The effect of cholesterol feeding, Roentgen ray treatment and pregnancy (Goat 17, — - —) and of cholesterol feeding, Roentgen ray treatment and castration (Goat 20, — — —). X indicates Roentgen ray treatment.

These observations seem to suggest that castration and cholesterol feeding both tend to increase the cholesterol content of the blood, but that the Roentgen rays, as has already been suggested, appear to have a depressing influence on the cholesterol values, the latter deduction being corroborated by the alternating effect of the week of Roentgen treatment (decrease) and the week of rest (increase) which became more marked during this period.

The fact that G26, receiving Roentgen treatment alone and having the lowest cholesterol value of all, appears to be slowly increasing his cholesterol percentage may perhaps be explained as follows. It is well known that the reproductive cells in the sex glands are particularly sensitive to the Roentgen rays and that animals can be castrated by Roentgen treatment alone. Prolonged exposure to the rays, even when extremely small doses are used, may therefore result in permanent injury, if not in the destruction of the reproductive cells.

It is possible that the total number of Roentgen ray units (approximately 150 milliampere minutes of diffuse treatment, wide open diaphragm) which G26 received in 5 months, may have sufficed to produce changes in the sex glands of the animal, and that the increase of the cholesterol values found in its blood are the first indication of these histologic changes. However, microscopic examination of the tissues will have to furnish conclusive evidence on this point.

The sudden drop observed in the cholesterol percentage of G16 on March 27 (from 0.302 to 0.216 per cent) may have been due to the effect of the Roentgen treatment during the week preceding this entry. However, as the animal had been lying down a good deal, appeared listless, and had been feeding badly for 3 days, the influence of a slight ailment followed by spontaneous and complete recovery must also be considered, especially as the cholesterol values remained constant during the following weeks.

A recent publication by Loewenthal (19) on the effect of castration on the blood of rabbits appears to corroborate my findings as regards the increase of the cholesterol content in the blood of goats.

The effect of pregnancy on the cholesterol values in women has been studied by Neumann and Herrmann (26), Aschoff (3), and Autenrieth and Funk (27); they report that an increase can be

demonstrated at the beginning of the 5th month. Bacmeister and Havers (2) observed a similar change in the bile of pregnant bitches. As far as could be ascertained no other data have been published on the subject.

In my experiment the changes found in the blood of G17 during pregnancy are difficult to account for, because of additional factors (*i.e.*, cholesterol feeding, Roentgen treatment). On the whole, the values seem to be slightly higher. That the increase is not more pronounced may be due to the Roentgen treatment, to which the organism might be particularly sensitive during pregnancy. As in other instances my observations on this point will have to be verified by future experiments.

In the light of recent studies on the stimulation of cell division by cholesterol, the influence of pregnancy and of castration on the cholesterol content of the blood would seem to be significant; in pregnancy the increase of the cholesterol values might be interpreted as a physiologic measure calculated to stimulate cell division in the embryo, while in castration elimination of the reproductive organs (in which cell proliferation is more constant than in any other part of the body) might be expected to result in a surplus of unused cholesterol.

The results indicated by my observations during the third period may be summarized as follows:

1. Anesthesia appears to reduce the cholesterol content of the blood.

2. Castration tends to increase the cholesterol values; the increase of body fat observed in castrates and the fact that cholesterol is stored in the body fat are in accordance with the apparent effect of castration.

3. The highest cholesterol values were found in the castrated and cholesterol-fed goat, the lowest values in the animal that had not been castrated and had received diffuse Roentgen treatment only, while intermediate values occurred in the animal that had been castrated and treated with Roentgen rays. These facts seem to support the assumption that castration increases and Roentgen treatment tends to reduce the cholesterol content of the blood.

4. The slight but steady increase of cholesterol found in the blood of the goat which received diffuse Roentgen treatment

alone recalls the fact that the Roentgen rays may destroy the reproductive cells to an extent equalling castration, and suggests that the increase itself may be due to the destruction of the reproductive cells in this animal. The observation will have to be corroborated by microscopic findings.

5. The influence of pregnancy on the cholesterol content of the blood, though indicated, is not sufficiently marked to permit definite conclusions. Whereas in the other goats cholesterol feeding and Roentgen treatment appeared to neutralize each other, it is possible that pregnancy rendered this animal more sensitive to the effect of the rays and that the latter factor preponderated and counteracted the influence of pregnancy. Future observations alone can furnish conclusive data.

Technical Details Concerning the Chemical Analysis of the Blood for Cholesterol.

The original Autenrieth-Funk (27) method was used at first to determine the cholesterol values of the blood in my experiments. Towards the middle of the second period parallel tests were made by Autenrieth's method and Bloor's (24) modification thereof, which was published at the time. Latterly Bloor's first modification has been used exclusively as it is much more simple and in many cases appears to give more accurate results. Bloor's (28) second modification has not been used; the omission of saponification in the latter method gives somewhat higher values, and it was thought advisable to retain one standard in this series of experiments.

The fault of Autenreith's method to which Bloor has called attention, the occurrence of a brownish tint in the reaction, making colorimetric comparison difficult, though also observed by me in samples of pathologic human blood, has not given me any trouble in the tests made with goat's blood. In the latter instances both methods gave identical values with only a few exceptions; when a difference occurred it never exceeded 0.025 per cent and the average values of a great number of readings by both methods have been recorded in the table (second period, Jan. 17 to Feb. 1).

The test for cholesterol is based on a specific color reaction;

on addition of acetic acid anhydride and sulfuric acid the colorless chloroform extract of the blood assumes a green tone, varying in intensity according to the amount of cholesterol present in the blood. The time required for the reaction, *viz.*, till the maximum color intensity is reached, in any test is 15 minutes, according to Autenrieth and Funk (27), and Bloor (24), the reagents being left in the dark at a constant temperature of 35–37°C. during that time. In the course of my investigations the following observations led me to modify the factors of time and temperature.

I found that the rapidity of the reaction varied considerably in different blood samples,[2] the maximum color value being reached much more quickly in one specimen of blood than in another under parallel conditions. As the exact chemical nature of the process by which the color reaction is caused is not known, the assumption seems warrantable that the presence of other chemical substances besides cholesterol may retard or accelerate the reaction. Every precaution has been taken to prevent recognizable factors (impurity of reagents, difference in temperature) from playing a part in the occurrence of these variations. To obtain data as accurate as possible by taking the occurrence of these variations into account, I have adopted the following procedure.

The test is left in the dark[3] at room temperature for 5 minutes only, after which it remains exposed to the light, and readings with the Autenrieth-Hellige colorimeter (control of the instrument with tests containing pure cholesterol) are made at intervals of 2 minutes until the maximum color intensity is reached. In some cases the maximum value was found after two readings, in others after ten to thirty readings; it remains constant for 10 minutes in some and for as long as 20 minutes in others, after which the color begins to fade, giving lower values. It is obvious

[2] I tested over 400 different blood samples during the last 6 months, the cholesterol value being determined in normal human blood. in human blood under various pathological conditions, and in the blood of several species of experimental animals (dogs, goats, spermophiles). An average of ten readings was made for each test, resulting in a total of 4,000 readings.

[3] I have found an ordinary microscope case very serviceable as a "dark cupboard."

that a test which reaches its maximum value after two readings or 4 minutes, and in which the maximum remains constant for 8 minutes only, will already have begun to fade when the first reading is made after 15 minutes, and that, therefore, the value obtained will be lower than would be warranted by the amount of cholesterol present in the blood. When the reaction is of the rapid type described above, it is advisable to make two, three, or even four tests with different portions of the same extract; the same maximum value will be constant for the same length of time in these different portions, and the figures recorded in the tables will represent maximum values which remained constant for at least four consecutive readings at 2 minute intervals each.

Another observation which caused me to adopt the above technique was that some tests assume a decidedly yellow-green tint (not the brown tone referred to by Bloor) when left in the dark longer than 5 minutes. This tint differs materially from the emerald green of the pure cholesterol reaction and makes accurate colorimetric reading difficult. I noticed, however, that the exact shade required for matching the control color (either pure cholesterol or the fluid of the Autenrieth-Hellige colorimeter) can be obtained by exposing yellowish tests to strong daylight (near a window); whereas tests that are slightly too blue-green can be corrected by putting them in the dark for a couple of minutes. The actual values are not affected by the change of tint.

DISCUSSION.

Although the data furnished by the observations reported in this paper are by no means conclusive, they seem to suggest problems worth studying, *viz.*, the effect of Roentgen rays on the cholesterol content of the blood in relation to malignant conditions, the influence of the organs of reproduction on cholesterol metabolism, and the effect of bacterial or parasitic infection. The fact that the primary object of my experiment was the study of possible factors in malignancy rather than the study of cholesterol values for themselves, will, I think, sufficiently explain the indeterminate nature of most of my deductions. It must be remembered also that the animals used for the experiment were young, healthy, and of a species endowed with a remarkable power of resistance and recuperation, having many

means of safety at their disposal in the organs that regulate cholesterol metabolism. The adequacy of these organs, moreover, is bound to remain an unknown quantity in every animal as long as the animal is alive. Since, comparatively speaking, the study of cholesterol metabolism is a new field of investigation, data for comparison are scarce, if they are not entirely lacking, as is the case in experimental work of this kind done on goats. However, although my experiment is still in progress, some of the results obtained so far by measures calculated to disturb the cholesterol balance seem to be encouraging, and I hope to publish these shortly.

SUMMARY.

1. The average normal cholesterol content of the blood in goats, though not definitely established, appears to be from 0.186 to 0.216 per cent.

2. The cholesterol content of the blood can be increased by cholesterol feeding.

3. Diffuse Roentgen treatment appears to lower the cholesterol content of the blood, but conclusive evidence will have to be furnished by future experiments. The beneficial effect of Roentgen treatment in malignant conditions, the increase of the cholesterol content observed in the blood of patients suffering from malignant disease, and the stimulation of cell division by cholesterol demonstrated by recent experiments, suggest that the study of the influence of the Roentgen rays on the cholesterol content of the blood may be of considerable value.

4. Castration tends to increase the cholesterol values, which may explain the increase of body fat found in castrates and after the cessation of reproductive activity, since the storage of cholesterol in the body fat has been demonstrated.

5. The increase of the cholesterol content of the blood in pregnancy, reported by others, has been found indicated in my experiment, although intercurrent factors forbade definite conclusions.

6. The normal process of growth in the embryo caused by cell proliferation and the effect of cholesterol on cell division referred to above may explain the increase of the cholesterol content of the blood during pregnancy.

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